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# ON THE CONTROL OF pH DURING EXPERIMENTS ON THE MODE OF ACTION OF SULFONAMIDES

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In a series of studies dealing with the mode of action of the sulfonamides (1-4), certain quantitative methods were described and employed. Unfortunately, no specific statements were made regarding the changes in pH during the course of the experiments and it is therefore the object of this note to establish their magnitude and possible bearing upon the conclusions reached.

We have measured with the glass electrode the pH changes induced by *Escherichia coli* (No. 6522, American Type Culture Collection) in three different media used previously. SG, containing inorganic salts and glucose, pH 7.2 before autoclaving; SGPP, being SG plus 1 per cent proteose peptone; PPFG, containing 2 per cent proteose peptone, glucose and fumaric acid, pH 7.8 before autoclaving.

TABLE 1

The pH of cultures inoculated with about 80 organisms per cc.

The sulfonamide concentrations were adjusted to inhibit the rate of growth by 50 per cent in this experiment:  $3 \times 10^{-3} M$  sulfanilamide and  $8 \times 10^{-3} M$  sulfathiazole.

MEDIUM	SULFONAMIDE	pH	
		Uninoculated control	132 million per cc.
SG	SA	7.05	6.85
		7.05	6.85
SGPP	ST	6.95	6.90
		6.95	6.85
PPFG	ST	7.7	7.5
		7.7	7.5

Eight cc. of medium was inoculated with about 150 bacteria, and the cultures incubated at 37°.

Medium containing enough sulfanilamide or sulfathiazole to inhibit the rate of growth from 25 to 75 per cent was also tested. Visible turbidity appeared in the peptone containing media in about seven hours, and in SG in about 16 hours, after which it could be followed by means of a photoelectric densitometer.

Typical results are shown in table 1. Until the time of just visible growth there is no appreciable change in pH. At the final population density used in all of our experiments (132 million per cc.) the drop is within 0.3 units, both in the presence and absence of sulfonamide. On the other hand the maximum changes obtained by allowing the cultures to grow as far as the media permit are much greater.

The small changes in the pH recorded above are further minimized in our ex-

periments, in that rates of growth are measured on the basis of how much time is required for a given inoculum, usually 20-100 bacteria per cc, to reach the end-point of 132 million per cc. Such a period of growth involves the production of about 20 generations. However, not until the sixteenth or seventeenth generation does growth become visible and any appreciable change in pH occur. Thus the small change in pH occurs for but a fraction of the entire period of growth. It is therefore concluded that the quantitative results and interpretations previously reported can not be questioned because the pH was inadequately controlled (5).

#### SUMMARY

Checking on methods previously used in studying the mode of action of the sulfonamides, it is shown that the changes in pH are within 0.3 unit. This is too small to affect the interpretation of the data.

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# THE RELATIVE RATES OF ABSORPTION OF QUININE AND SOME OTHER CINCHONA SALTS FROM ISOLATED INTESTINAL LOOPS OF DOGS

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In a recent communication from this laboratory (1) there were reported some results on the rates of absorption of quinine sulfate and quinine dihydrochloride from isolated Thiry Vella intestinal loops of dogs. In the present paper we wish to report an amplification of this study with four other animals, together with comparisons of the rates of absorption of quinine as the sulfate the dihydrochloride and the free base and with the dihydrochlorides of cinchonidine cinchonine, dihydroquinidine and totaquine. In addition, we wish to report a more detailed investigation of the effect of sodium chloride on the solubilities of quinine cinchonine and cinchonidine sulfates and on the lack of effect of this increase in solubility on the rate of absorption of the quinine sulfate.

**METHODS AND RESULTS** Dogs with jejunal loops were prepared through the courtesy of Drs H M Schiebel and Max Rogers by the method described by Johnston (2). All loops were 25 to 27 cm in length. The technique used in the absorption measurements was identical with that described in our earlier paper (1) and the residual quinine was, as before, determined by the method of Kyker, Webb and Andrews (3). In the case of absorption experiments with other cinchona compounds the modification of the above method described by Lewis (4) was employed.

As a means of securing a more quantitative removal of the cinchona compound especially the sulfate, from the loop we have again employed physiological saline solution. The ability of this solution to dissolve much larger amounts of quinine sulfate than does pure water previously reported by Andrews and Anderson (1) has been confirmed and the solubility curve in sodium chloride has also been determined for cinchonine and cinchonidine sulfates. Some possible physiological implications of this solubility effect are discussed below. The solubility determinations were made in the same way as previously described using pure samples of the compounds concerned. The results are recorded in table 1. It will be observed that the results for quinine sulfate are in substantial agreement with those published by Andrews and Anderson but the present data have more closely defined the maximum of the curve. The solubility curves of cinchonine and cinchonidine sulfates are of similar form. Quinine free base, on the other hand shows only a very slight change (a decrease) in solubility with increasing concentrations of sodium chloride. It seems obvious that this sodium chloride solubility effect is confined to the cinchona sulfates.

Table 2 shows the relative rates of absorption of quinine sulfate, quinine dihydrochloride and quinine free base for various periods of time from the four dogs employed. In each case the standard dosage used was equivalent to 20.0 mgm of anhydrous quinine sulfate (or 17.36 mgm as the free base)

TABLE 1

*Solubility of various Cinchona compounds in increasing concentrations of sodium chloride at 40°C*

(Both concentrations expressed as grams per 100 ml solution)

CINCHONA COMPOUND	CONC. OF NaCl	CONC. OF CINCHONA (AS FREE BASE) PER 100 ML. OF SOLUTION
Quinine sulfate	0.0	0.2625
	0.4	0.3200
	0.9	0.3900
	1.5	0.4737
	2.0	0.5612
	2.5	0.5812
	3.5	0.6512
	4.0	0.6850
	4.2	0.5462
	4.5	0.4700
	5.0	0.3787
Quinine base	0.0	0.0251
	0.4	0.0242
	0.9	0.0230
	1.5	0.0235
	2.0	0.0227
	4.0	0.0184
Cinchonidine sulfate	0.0	1.4550
	0.4	1.9375
	0.9	2.1775
	1.5	2.5825
	2.0	1.5000
	4.0	1.0425
Cinchonine sulfate	0.0	1.6250
	0.4	2.0000
	0.9	2.4000
	1.5	2.2500
	2.0	2.3000
	4.0	1.7000

per kgm of body weight. Whereas the sulfate was introduced into the loop in water suspension, the dihydrochloride was dissolved in 20 cc water. Results are recorded for 30, 60 and 90 minute absorption periods and individual figures are recorded in order to show the order of experimental variation. In some of the later tables, where other conditions are introduced, the average of some of the groups of figures in table 2 are included for convenience in com-

TABLE 2

*The absorption of quinine as the sulfate the dihydrochloride and the free base from isolated intestinal loops of dogs*

All doses are in terms of 20 mgm anhydrous quinine sulfate per kgm body weight

LENGTH OF EXPT.	PERCENTAGE OF QUININE ABSORBED			
	Dog I 14.2 kg	Dog II 22.3 kg	Dog III 20.3 kg	Dog IV 11.6 kg
Quinine sulfate				
min.				
30	21.8	15.8	39.0	60.4
30	25.8	21.4	47.1	65.1
30	17.1	22.4	56.7	77.6
60	36.4	34.8	66.6	67.7
60	35.2	21.5	59.5	89.1
60	26.7	23.3	60.0	70.3
90	26.9	25.3		
Quinine dihydrochloride				
30	32.7	34.4	52.9	90.0
30	21.8	27.8	50.6	68.6
30	23.2	30.4	50.9	83.1
60	32.1	39.8	77.2	97.0
60	28.3	42.2	67.2	95.3
60	39.6	40.2		
90	39.4	46.9		
Quinine free base introduced in suspension as such				
30	18.3	15.0		
30	21.6	16.7		
60		25.6		
60		17.0		
Quinine sulfate with equivalent sodium hydroxide				
30	16.1	15.6		
30	8.8	19.7		
30	9.4			
30	13.7			
60	21.0			
60	17.7			
Quinine dihydrochloride with equivalent sodium hydroxide				
30	17.4	24.6		
30		18.8		
Quinine dihydrochloride with equivalent sodium carbonate				
30	20.8	31.4		
30	18.~	35.3		

parison In this table absorption figures are expressed only in terms of percentage of the cinchona compound absorbed Blank determinations were made with all animals to demonstrate the extent to which the cinchona compound could be quantitatively removed from the loop immediately after its introduction As stated by Andrews and Anderson, these recoveries usually varied from 90 to 98% of the drug

The data in table 2 illustrate several generalizations concerning the mechanism of absorption It is obvious that considerable difference would be expected to exist between individual animals Even if two dogs of the same age and weight were used to prepare two loops of exactly the same position and length, individual differences in absorptive speed would certainly result It will be observed that these speeds vary considerably for some of the present animals from the speeds reported by Andrews and Anderson (1) As a result, each individual animal must be standardized by some uniform procedure We prefer, for this purpose, to use the percentage absorption obtained from quinine dihydrochloride in a 30 minute experiment Because of its greater solubility, the dihydrochloride is a more convenient means of standardization than the sulfate It is evident that wide differences obtain between the absorption speeds of these four dogs Dog IV absorbs either the sulfate or the dihydrochloride at about three times the speed of Dog I Furthermore, in comparing for any one dog, the amount absorbed in 30 minutes with that absorbed in 60 to 90 minutes, it is evident that no linear relation exists between percentage absorption and time The marked flattening of the resulting curve would be inescapable if the percentage absorption were approaching 100% and would mean nothing more than exhaustion of the material being absorbed But this flattening of the curve is evidenced with those dogs which absorb only about one-third of the dose in 60 minutes When left in the loop for an additional 30 minutes no further significant amount is absorbed

We have considered the possibility of a preferential absorption of the acid combined in the salt, leaving behind the free base with its obviously slower speed of absorption However, the data in table 2 show that even the free base is absorbed in 30 to 60 minutes at too rapid a rate to account for the practically static condition prevailing with the salt from 60 to 90 minutes Moreover, the sharp differences which have long been noted between the rates of absorption of inorganic chlorides and sulfates are not reflected in our data The slope of the curve of absorption versus time decreases as much with quinine sulfate as with quinine dihydrochloride In addition to the above we have carried on absorption experiments for 60 minutes with the dihydrochloride in which extra acid (in the form of ammonium chloride) was introduced into the loop at the 30 minute point The ammonium chloride produced a pH value within the loop of approximately 5.0 In no case was any increase observed over the figures listed in table 2 for 60 minutes However, the fact that the loop was not damaged by this treatment was shown by its ability to produce normal absorption values on subsequent standard measurements

The prevailing pH of jejunal loop contents was found by Robinson, Lucky

and Mills (5) to range from 6.13 to 6.94. We have found, under normal conditions, pH values of as high as 7.11 for the contents of our loops. In one case after a dose of quinine dihydrochloride had remained in the loop for 30 minutes the removed contents showed pH 6.60. If one calculates from the constants of Christophers (6) for quinine the fraction present as free base at these pH values, he finds at pH 7.11 about 6% and at pH 6.60 about 2% of the total charge. On these grounds alone it would seem unlikely that a sufficient proportion of the salt administered would be converted to the free base in order that the somewhat slower absorption of the latter could account for the form of the curve of absorption with time. In this connection it is interesting to speculate as to whether at the prevailing pH of these loops any free base introduced is largely converted to salt before being absorbed. Such a reaction might, due to the insolubility of the free base, be very sluggish. It is to be noted that when free base was presumably produced in the loop by simultaneous addition of an equivalent amount of sodium carbonate to quinine dihydrochloride one animal (Dog II) showed about the same rate of absorption as for the unneutralized dihydrochloride. It is evident that reactions of this sort in the loop are capable of being very sluggish. One is almost compelled to postulate some type of physiological fatigue to explain the much decreased rate of absorption after the first or second period of 30 minutes. The known effect of quinine on smooth muscle may well cause such an effect.

The quinine free base when introduced as such, was ground to a powder sufficiently fine to permit its being forced through the rubber tube into the loop without choking the former. When we attempted to produce the free base by putting into the loop either sulfate or dihydrochloride with equivalent amounts of alkali the quinine salt was first put in and followed at once with the alkali.

A practice recommended by some clinicians of administering therapeutic doses of hydrochloric acid along with quinine sulfate should be mentioned. The amounts of acid used could hardly be capable of lowering significantly the pH of either normal gastric contents or of those of the small intestine (7) but could contribute equivalent amounts of sodium chloride to the latter. In view of the marked increase in solubility of quinine sulfate caused, up to a certain point, by increasing concentrations of sodium chloride it was thought worth while to test the effect of sodium chloride on the rate of absorption of quinine sulfate. The results are recorded in table 3 in which is included for comparison, an average figure taken from table 2 for the absorption of the quinine salt from water solution.

The results in table 3 show that in spite of the fact that in 0.9% sodium chloride quinine sulfate is about 50% more soluble than in pure water its rate of absorption is not significantly increased. On the assumption that this amount of sodium chloride, added to that naturally present may not be significant, we have tested the rate of absorption from that solution giving the maximum solubility of quinine sulfate (4%, see table 1). This very hypertonic solution is no doubt rapidly diluted both by intake of water and loss of sodium

chloride and it was thought possible that in the course of this process a point would be reached where any favorable effect of the salt might demonstrate itself. However, as the data show, the only result was with both dogs a small decrease. We may conclude that the mere matter of physical solubility of the salt is without influence on its rate of absorption and that the attractive idea of raising the speed of absorption of quinine sulfate by the use of sodium chloride is without experimental support.

The results of the application, conversely, of sodium sulfate solution to the absorption of quinine dihydrochloride show some diminution in speed of ab-

TABLE 3

*Influence of sodium chloride and other salts on absorption of quinine sulfate, dihydrochloride and free base*

Doses identical with those in table 2

QUININE SALT USED	CONDITIONS	LENGTH OF EXPT <i>min</i>	PERCENTAGE ABSORBED	
			Dog I	Dog II
Quinine sulfate	Water suspension	60	33.7 (av)	26.5 (av)
	0.9% sodium chloride	60	35.8	29.9
		60	36.2	26.6
	4.0% sodium chloride	60	29.9	23.3
Quinine dihydrochloride	Water solution	30	25.9 (av)	31.1 (av)
	0.9% sodium chloride	30	31.7	23.8
	Sodium sulfate equimolar to 0.9% sodium chloride	30	17.7	21.1
		30	23.4	27.5
	Sodium sulfate equimolar to the quinine	30	26.2	31.4
		30	28.2	28.6
Quinine free base	Water suspension	30	19.9 (av)	33.3 (av)*
	0.9% sodium chloride	30	15.9	30.6
		30	19.3	32.9
	Sodium sulfate	30	18.9	28.5
	Equimolar to the quinine	30	25.0	32.9

\* Quinine base made by action of sodium carbonate on equivalent quinine dihydrochloride

sorption when the more concentrated sodium sulfate solution was used. The effect however, appears to be slight. Neither sodium chloride nor sodium sulfate has any appreciable effect on the absorption of the free base.

The therapeutic employment of some of the other more common cinchona compounds as schizonticidal agents makes their comparative rates of absorption a matter of interest. We have therefore used Dogs I and II of this study for measurements on the dihydrochlorides of cinchonine, cinchonidine, dihydroquinidine and the commercial mixture known as "totaquine." The composition of the latter has recently been recorded by Seeler, Dusenbery and Malanga (8). These investigators found little difference in the schizonticidal activity of

quinine, quinidine, cinchonine and cinchonidine, from which one might conclude that sharp differences in absorptive speed would be unlikely. Our results, recorded in table 4, support this assumption. Quinidine was not included in this study because of its failure to respond satisfactorily to the silicotungstate method of determination. In table 4 the average figures for quinine dihydrochloride for both 30 and 60 minute periods in both dogs are included for convenience in making comparisons.

The data in table 4 are of interest in connection with the observations of Hiatt (9) who has recently reported on the plasma concentrations of the four common cinchona alkaloids resulting from administration by mouth of the free bases. As compared with quinine, quinidine and cinchonidine which

TABLE 4

*Absorption of the dihydrochlorides of cinchonine, cinchonidine, dihydroquinidine and totaquine in water from isolated intestinal loops of two dogs*

Dose of each salt equivalent to 20.0 mgm. anhydrous quinine sulfate per kg. body weight (table 2)

CINCHONA COMPOUND USED (DIHYDROCHLORIDE)	LENGTH OF EXPERIMENT  min	PERCENTAGE ABSORBED	
		Dog I	Dog II
Quinine	30	25.9 (av.)	31.1 (av.)
	60	33.6 (av.)	40.7 (av.)
Cinchonine	30	41.2	38.2
	60	57.5	64.0
Cinchonidine	30	36.2	16.3
	60	42.7	35.6
Dihydroquinidine	30		34.4
Totaquine	30		35.6

gave figures of comparable order, cinchonine was reported as outstanding by reason of its having produced plasma values so low as to be almost negligible. These results are obviously at variance with ours listed above, for this alkaloid. However, it should be noted that we used cinchonine dihydrochloride in our measurements of the speed of its disappearance from the loop whereas Hiatt administered the alkaloid as the free base. Judging from our results with quinine, we should expect the free base to be much less rapidly absorbed than the dihydrochloride. The possibility of differences in the rôle of metabolic destruction should also be considered. We plan to investigate further the apparent difference between these results.

#### CONCLUSIONS

1. Data are presented showing the effect of increasing concentrations of sodium chloride on the solubility of quinine, cinchonine and cinchonidine sul-

fates and on quinine free base. Each of the above sulfates shows increasing solubility as the sodium chloride content of the solution increases, with a maximum solubility at a sodium chloride percentage which varies with the compound studied. No such curve is obtained with quinine free base, in this case the sodium chloride is practically without effect.

2 The relative rates of absorption of quinine sulfate, quinine dihydrochloride and quinine free base from isolated intestinal loops of four dogs are recorded for varying periods of time.

3 The amount of the compound absorbed does not bear a linear relationship to time, even when an ample supply remains in the loop. The rate of absorption greatly decreases during the second and third half-hour of the experiment. More rapid preferential absorption of the acid combined with the alkaloid is not a valid explanation of this effect.

4 Although sodium chloride, in concentrations up to 4%, markedly increases the solubility of quinine sulfate, the presence of sodium chloride in the loop causes no increase in the rate of absorption of the alkaloid.

5 Data are presented on the relative rates of absorption of the dihydrochlorides of cinchonine, cinchonidine, dihydroquinidine and "totaquine" from the loops of the dogs used in this work. Very little significant difference exists between the rates of absorption of any of these compounds and quinine dihydrochloride, although the highest figures were obtained with cinchonine.

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# THE IN VITRO BACTERIOSTATIC ACTION OF SOME SIMPLE FURAN DERIVATIVES

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Several previous bacteriological investigations have been reported on the properties of certain furan compounds. McGuigan (1) found a phenol coefficient of 0.26 for furfural using *E. typhi* as the test organism. According to Kaufman (2), furoic acid (0.5%) was germicidal for *E. coli* in five minutes and higher concentrations had the same action on *S. aureus*, but salts of the acid were inactive. He concluded that the antibacterial properties of furoic acid resembled those of benzoic acid. A further study of the bacteriological properties of furoic acid by Calloway, Gilman and Werkman (3) showed that several compounds in a series of alkylated furoic acids were germicidal. Phatak and Leake (4, 5) reported that 2-furan mercurial compounds, furan compounds containing carboxyl groups, and condensation compounds of furan and phenol were all bactericidal.

The present report represents an investigation of the bacteriostatic action of some simple derivatives of furan, most of which have not been previously studied in this respect. Preliminary work revealed that compounds containing a nitro group attached to the furan nucleus were bacteriostatic. An extensive series was prepared and in many cases, the non nitrated analogue was included for comparison.

The compounds listed in tables 1, 2, 3 and 4 may be conveniently considered as derivatives of four simple furan compounds. This view was employed in deciding on the series to be prepared for this study and served to establish a system for the evaluation of the compounds. The four simple compounds chosen were furan, 2-furaldehyde, 2-furfuryl alcohol and 2-furoic acid. Ethyl- $\beta$ -furoacrylate was included in the table with the other derivatives of furoic acid because it was the only derivative of 2-furylacrylic acid tested.

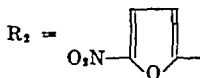
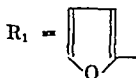
Forty-two compounds were prepared and tested for their bacteriostatic action. Twenty-five contain a nitro group in the 5-position. Seventeen of these were compared with the corresponding simple derivatives with a hydrogen atom in the 5-position. Since 2-nitrofuran still has the 5-position occupied by a hydrogen atom, 2,5-dinitrofuran was also prepared. Methods of synthesis of a majority of the compounds have been described previously. The compounds listed below which have not been previously reported were prepared by methods already described for similar compounds.

**EXPERIMENTAL.** Strains of *Staphylococcus aureus*, *Streptococcus hemolyticus*, *Diplococcus pneumoniae* type I, *Escherichia typhi*, *Escherichia coli* and *Pseudomonas pyocyanea* were employed as the test organisms. All tests were done in beef infusion broth containing 10 percent normal horse serum for tests on the pneumococcus.

TABLE 1

*Bacteriostatic action of some derivatives of furan*

Minimum bacteriostatic concentration in mgm % after 24 and 96 hours incubation

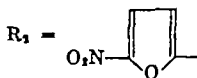
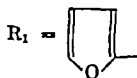


COMPOUND	S. AUREUS		STR. HEMOLYTICUS		D. PNEU. MONIAE I		E. TYPHI		E. COLI		PS. PYOCYANEA	
	24	96	24	96	24	96	24	96	24	96	24	96
I $R_1-H$	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
II $R_1-C_2H_5$	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
III $R_1-CO-CH_3$	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
IV $R_1-H$	10.0	20.0	5.0	20.0	5.0	10.0	1.25	2.5	1.25	2.5	2.5	40
V $R_1-NO_2$	2.5	5.0	1.25	5.0	10.0	>100	1.25	10.0	5.0	10.0		
VI $R_1-C_2H_5$	10.0	40.0	10.0	40.0	10.0	10.0	5.0	10.0	5.0	20.0	40.0	100
VII $R_1-CO-CH_3$	0.5	1.66	0.5	1.66	0.2	1.25	0.5	1.0	1.0	1.0	20.0	20
VIII $R_1-CO-C_2H_5$	1.25	2.5	1.0	2.5	1.0	>20.0	1.0	2.5	2.5	5.0		
IX $R_1-CO-C_2H_5$	1.0	1.25	0.5	2.5	0.2	>20.0	2.5	10.0	5.0	10.0		
X $R_1-CO-C_6H_{11}$	0.5	0.5	0.5	2.5	5.0	>10.0	2.5	5.0	2.5	2.5		
XI $R_1-CO-C_6H_{11}$	1.0	1.0			2.5	>2.5	2.5	2.5	>2.5	>2.5		

TABLE 2

*Bacteriostatic action of some derivatives of 2-furaldehyde*

Minimum bacteriostatic concentration in mgm % after 24 and 96 hours incubation



COMPOUND	S. AUREUS		STR. HEMOLYTICUS		D. PNEU. MONIAE I		E. TYPHI		E. COLI		PS. PYOCYANEA	
	24	96	24	96	24	96	24	96	24	96	24	96
XII $R_1-CHO$	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
XIII $R_1-CH=N-NHCONH_2$	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
XIV $R_1-CH=OH-C_2H_5$	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100		
XV $R_1-CHO$	1.25	1.25	2.5	10.0	5.0	40.0	1.0	1.0	2.5	2.5	10.0	20
XVI $R_1-CH=N-NHCONH_2$	1.0	1.25	1.25	5.0	2.5	>20.0	0.5	1.0	1.0	1.25	>20.0	>20
XVII $R_1-CHOH-C_6H_5$	10.0	20.0	10.0	100	20.0	100	0.5	0.5	2.5	5.0		
XVIII $R_1-CH=NOH$	5.0	10.0	2.5	5.0	100	100	0.5	0.5	1.0	1.0		

The compounds were dissolved in sterile beef infusion broth by warming and filtered through sterile Berkefeld filters. Ten cc quantities of the desired concentrations in broth were placed in sterile culture tubes for testing. Compounds were tested up to their maximum solubility in broth where this did not exceed 100 mgm %. Substances more soluble were arbitrarily limited to a maximum test concentration of 100 mgm. %. In this manner no compound lowered the pH of the test broth much below neutrality which would have occurred if furoic or nitrofuroic acid had been used in higher concentrations.

The inoculum consisted of organisms from eighteen hour cultures in beef infusion broth containing 10 percent normal horse serum for the pneumococcus and streptococcus. The size of the inoculum for the tests listed in the following tables was 10,000 to 20,000 organisms. Incubation was carried out for four days at 37°C with daily observations of the results. Estimations of bacteriostatic action were made by visual examination of the test medium for lack of growth or by comparison of the turbidity caused by growth in the medium containing the test substances with appropriate control cultures. The twenty four hour readings in the following tables represent the minimum concentration of compound which limited growth to fifty per cent of normal growth in that time period. The ninety-six hour readings denote the minimum concentration capable of preventing any visible growth during the four day period.

The data in tables 1, 2, 3 and 4 demonstrate the value of the nitro group for the bacteriostatic action of simple derivatives of furan. Of the seventeen non-nitrated furan compounds tested only three—compounds XX, XXI and XXII in table 3, the acetic, propionic and butyric acid esters of 2-furfuryl alcohol—were bacteriostatic under the test conditions. The addition of a nitro group in the 5-position in the fourteen inactive compounds conferred definite bacteriostatic properties on them and in general, increased the activity of the three non-nitrated alcohol esters mentioned above. Also, seven more nitrofuran derivatives tested all showed bacteriostatic action. Only one such derivative of the total of twenty five tested was inactive, compound XXXIX, table 4, *n*-butyl 5-nitro-2-furoate which may have been due to its low solubility in broth which was less than 1.5 mgm %. It was tested only as a saturated solution which was not bacteriostatic. This compound was the single exception to the general activity in the nitrofuran series. The bacteriostatic effect produced by the addition of a nitro group to the inactive furan compounds in this series was the least pronounced with furoic acid. Compound XXV, nitrofuroic acid exerted a bacteriostatic effect on four of the six strains tested only at the maximum concentration employed 100 mgm %, and was inactive on *Ps. pyocyanea*. Actually further tests not given here showed that both furoic acid and nitrofuroic acid in concentrations of 150 mgm % were bacteriostatic but such concentrations lower the pH of the test broth considerably below the optimum range for growth which may be responsible for their bacteriostatic properties at these concentrations.

As a group the nitrofuran compounds were least effective in inhibiting the growth of *Ps. pyocyanea*. In general, they effectively limited the growth of the other five species tested, although the minimum bacteriostatic concentration of the individual compounds for the various species varied considerably. Several compounds for example, VII, VIII, IX and X in table 1, XVI in table 2 and XL in table 4 were consistently active against all five species in low concentrations.

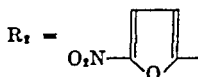
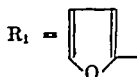
Compound XLII, 5-nitro 2-dibutyl furamide was the only compound in the series which was active on the Gram positive cocci but ineffective at its maximum concentration in broth on the Gram negative forms tested

A further consideration of the nitro compounds will lead to the general conclusion that the minimum bacteriostatic concentration of the ketone derivatives of furan in table 1 and the derivatives of 2-furaldehyde in table 2 are on the whole lower for these five strains than are the derivatives of 2-furoic acid in table 3 and 2-furfuryl alcohol in table 4. Furthermore, with the exception of

TABLE 3

*Bacteriostatic action of some derivatives of 2-furfuryl alcohol*

Minimum bacteriostatic concentration in mgm % after 24 and 96 hours incubation



COMPOUND	S. AUREUS		STR. HEMOLYTICUS		D. PNEU. MONIAE I		E. TYPHI		E. COLI		PS. PYOCYANEA	
	24	96	24	96	24	96	24	96	24	96	24	96
XIX $R_1-CH_2OH$	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100		
XX $R_1-CH_2OOCCH_3$	100	100	100	100	>100	>100	100	100	100	100		
XXI $R_1-CH_2OOCCH_2H_5$	25.0	50.0	10.0	20.0	100	100	25.0	100	20.0	25.0	25.0	100
XXII $R_1-CH_2OOCCH_2H_7$	20.0	20.0	20.0	>20.0	>20.0	>20.0	>20.0	>20.0	>20.0	>20.0		
XXIII $R_1-CH_2OCH_3$	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100		
XXIV $R_2-CH_2OH$	5.0	10.0	1.25	10.0	2.5	40.0	0.2	0.5	0.5	0.5	2.5	20
XXV $R_2-CH_2OOCCH_3$	20.0	100	5.0	100	10.0	>100	1.0	2.5	5.0	5.0		
XXVI $R_2-CH_2OOCCH_2H_5$	20.0	25.0	20.0	50.0	5.0	20.0	2.5	5.0	10.0	10.0	100	100
XXVII $R_2-CH_2OOCCH_2H_7$	20.0	20.0	5.0	20.0	1.0	10.0	2.5	2.5	20.0	20.0		
XXVIII $R_2-CH_2OCH_3$	10.0	20.0	5.0	20.0	5.0	20.0	1.0	1.0	2.5	2.5		

nitro furoic acid itself, the nitro derivatives of furoic acid are generally more effective than the alcohol derivatives. Various single exceptions to these generalizations are apparent from a thorough study of the four tables.

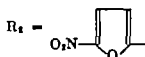
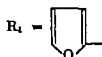
The twenty-four hour readings recorded in the preceding tables representing inhibition of growth to at least fifty per cent of growth in control cultures are clearly a measure of the bacteriostatic activity of the nitrofuran compounds. For a majority of the compounds, however, the ninety-six hour readings indicate that a higher concentration is required for complete inhibition of growth for that time period. This failure of growth to occur after such prolonged incubation suggested that the higher concentrations of some of the nitrofuran compounds

were bactericidal. This fact was determined with three of the compounds by subculture from the drug broth mixtures in which the concentrations were sufficient to prevent the appearance of turbidity for four days. In all three cases, no growth occurred in the subcultures after incubation. It was thus shown that 5-nitro-2-

TABLE 4

Bacteriostatic action of some derivatives of 2-furoic acid

Minimum bacteriostatic concentration in mgm % after 24 and 96 hours incubation

[illegible]

furyl methyl ketone (VII) 5-nitro-2 furaldehyde (XV) and 5-nitro-2-furfuryl propionate (XXVI) were also bactericidal since they were capable of sterilizing the inoculum which had been added. This point was more clearly indicated with 5-nitro-2-furfuryl propionate and in addition some idea of the time required for this sterilizing action was determined in the following experiment

The compound was mixed in broth as described previously and in the concentrations shown in table 5. Broth was used as a control. All tubes were in

oculated as indicated from an eighteen hour culture of *S. aureus* and at intervals up to forty-eight hours, 1 cc quantities were removed, and after proper dilution, added to and mixed well with nutrient agar in Petri dishes. After forty-eight hours incubation colony counts were made.

The results of the above experiment shown in table 5 substantiate the previous proof that 5-nitro 2-furfuryl propionate is bactericidal as well as bacteriostatic. Concentrations of 25 mgm % or more, completely sterilized the medium in twenty-four to forty-eight hours, while 20 mgm % merely inhibited growth for twenty-four hours.

It seemed impractical to attempt a determination of the bactericidal properties of all the compounds tested because of the time and effort required to apply either of the above tests to each compound. In view of these results it was assumed that concentrations of nitrofurans which completely inhibited growth for ninety-six hours were bactericidal.

TABLE 5  
*Bactericidal effect of 5-nitro-2-furfuryl propionate on Staphylococcus aureus*

CONCENTRATION mgm %	NUMBER OF ORGANISMS PER CC (HOURS)				
	0	4	8	24	48
100	210	20	30	none	none
50	200	50	20	none	none
25	280	750	1,210	10	none
20	250	2,500	2,960	112,000	>100 million
Control broth	300	620,000	100 million	>100 million	>100 million

The unfavorable effect of a large inoculum on the *in vitro* antibacterial action of certain types of chemical compounds suggested the study of this factor on the bacteriostatic and bactericidal action of nitrofurans. The experimental procedure was the same as described earlier for the bacteriostatic examination of these compounds except for the size of the inoculum. In this experiment, the inoculum consisted of 0.1 cc of an eighteen hour culture of five of the same organisms employed previously. Ten of the nitrofurans compounds listed in tables 1, 2, 3 and 4 were tested under these conditions, the four types of derivatives being represented by at least two compounds.

The results of this experiment listed in table 6 demonstrate the effect of the inoculum size on the bacteriostatic action of nitrofurans. A comparison of these data with the results in tables 1, 2, 3 and 4 shows that in almost every instance, a greater concentration of the nitro derivatives of furan, 2-furaldehyde and 2-furfuryl alcohol was required to exert a bacteriostatic effect. The action of nitrofuroic acid and its ethyl ester was abolished under the conditions of this test even at their maximum concentration in broth. In addition, the maximum broth concentration of ethyl 5-nitro- $\beta$ -furaerylate failed to show bacteriostatic action on *E. coli* when a large inoculum was employed, although it was

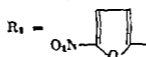
bacteriostatic for the other four test organisms. From these results it is apparent that a large inoculum distinctly reduced the bacteriostatic action of nitrofurans derivatives, especially so on nitrofuroic acid and its derivatives. It has already been pointed out that nitrofuroic acid was among the least effective of all the derivatives in the original test so that the loss of activity noted above with this compound was not as great as with the other two compounds which were effective in the original test at much lower concentrations.

A further study of the bacteriostatic properties of furan derivatives was made employing *E. coli* grown in a synthetic medium devised by Bliss and Long (6)

TABLE 6

Effect of a large inoculum on bacteriostatic action of ten nitrofurans compounds listed in tables 1, 2, 3 and 4

Bacteriostatic concentration in mgm. % after 24 and 96 hours incubation



COMPOUND	S. AUREUS		S. HEMOLYTICUS		D. PHOSPHORUM		S. TYPHI		E. COLI	
	24	96	24	96	24	96	24	96	24	96
IV $R_1-H$	10.0	40.0	18.0	30.0	10.0	20.0	5.0	10.0	10.0	30.0
VI $R_1-CaH_2$	40.0	100	40.0	40.0	40.0	40.0	5.0	10.0	30.0	30.0
VII $R_1-CO-CH_3$	5.0	10.0	2.5	5.0	5.0	10.0	1.0	2.5	10.0	30.0
XV $R_1-CHO$	10.0	10.0	10.0	10.0	5.0	100	1.25	2.5	5.0	30.0
XVI $R_1-CH=N-NHCONH_2$	2.5	5.0	5.0	10.0	20.0	>30.0	1.25	2.5	2.5	5.0
XXIV $R_1-CH_2OH$	10.0	>100	10.0	>100	5.0	100	1.0	1.0	10.0	40.0
XXVI $R_1-CH_2COOC_2H_5$	20.0	50.0	30.0	30.0	10.0	30.0	5.0	20.0	10.0	30.0
XXXV $R_1-COOH$	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
XXXVII $R_1-COOC_2H_5$	>20.0	>20.0	>20.0	>20.0	>20.0	>20.0	>20.0	>20.0	>20.0	>20.0
XL $R_1-CH=CH-COOC_2H_5$	2.5	5.0	1.0	5.0	2.5	>5.0	1.0	>5.0	>5.0	>5.0

In this test, all the compounds previously mentioned were dissolved in the medium in suitable concentrations and inoculated with approximately 20,000 organisms from an eighteen hour culture of *E. coli* which had been maintained constantly in this medium. The tests were incubated and read as already described.

The non nitrated furan compounds which were inactive in broth showed no antibacterial activity in this test. Of the two non nitrated derivatives of furfuryl alcohol which were active on *E. coli* in broth the activity of only one 2-furfuryl propionate was increased in the synthetic medium. The bacteriostatic action of the nitrofurans compounds on *E. coli* was enhanced in the synthetic medium with but one exception 5-nitro-2-furfuryl alcohol. 5-nitro-2-dibutyl furamide was not bacteriostatic for this organism in broth or the synthetic

medium The activity of the nitrofurans was increased on the average from two to ten fold while the most remarkable enhancement occurred with di-nitro furan which gave a fifty-fold increase As far as could be determined by an examination of the cultures and counts of viable organisms, the growth in the synthetic medium was not inferior to cultures in broth It was therefore suggested that the enhancement of the bacteriostatic activity of nitrofurans compounds in this medium is due to the absence of specific substances present in broth which interfere with the activity of the compounds A search for such substances is now under way

#### SUMMARY

A survey of the *in vitro* bacteriostatic action of forty-two furan compounds, including derivatives of furan, 2-furaldehyde, 2-furfuryl alcohol and 2-furoic acid has been presented It disclosed that with a few exceptions, the presence of a nitro group in the 5-position of the furan ring conferred considerable bacteriostatic action on such derivatives Twenty-five compounds of this type were tested and twenty-four showed bacteriostatic activity in varying degrees The single ineffective compound was tested as a saturated solution in broth which contained less than 1.5 mgm % The specificity of this group in activating these simple furan compounds was indicated by a comparison of seventeen nitrofurans compounds with their non-nitrated analogues Fourteen of the latter, entirely inactive under the test conditions were bacteriostatic when a nitro group was introduced into the 5-position, while the action of three slightly active derivatives was considerably enhanced by the same process The action of the nitro group was least effective with nitrofuroic acid The bacteriostatic action of the nitrofurans compounds was effective against both Gram positive and Gram negative organisms, being least active in inhibiting the growth of *Ps. pyocyanea* An examination of the antibacterial action of several of the nitrofurans compounds by subculture and plate count methods showed them to be slowly bactericidal in high concentrations, while lower concentrations were merely bacteriostatic for twenty-four hours or longer Both of these activities were decreased, and in some cases, completely abolished when large inocula were employed The enhancement of antibacterial action when tested in a synthetic medium suggested the existence of specific substances in broth capable of inhibiting both the bacteriostatic and bactericidal action of the nitrofurans complex

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# A SIMPLE METHOD FOR THE ROUTINE SEARCH FOR ANTIBIOTIC SUBSTANCES PRODUCED BY MOLDS

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Since 1929 when Fleming (1) first noted the production of an antibiotic substance by *Penicillium notatum*, many similar mold products have been observed by other workers. However until the work of Wilkins and Harris (2) in 1942, no systematic search for such substances was reported. At the time of their first paper, covering 100 fungal species, these authors did not feel justified in giving the details of the method used. Their second report (3) on another 100 fungal species refers to the method which was published shortly thereafter (4). Waksman and Horning (5) have reported on the antibiotic activity of a large number of antagonistic fungi isolated from soils, manures, composts and other natural substances. Atkinson (6) has since presented a similar study on 39 penicillia isolated from fruits and other sources. In a subsequent paper (7) her methods are described in detail.

Wilkins and Harris have suggested two qualitative methods. The first is to "grow the fungus to be tested on an agar plate and when the colony is about one inch diameter, to flood the plate with a suspension of the bacteria to be inhibited and incubate at 37 C. Any inhibitory substance produced by the fungus diffuses into the agar and there is a clear bacteria free zone around the edges of the colony." We have found the following objections to this method: (a) some fungi grow so rapidly that they may cover the entire plate before reaching the stage in their development when an antibiotic substance is discharged. (b) in at least one instance we have obtained using this method a positive test on one day followed by a negative on the next. Apparently the substance produced was rapidly destroyed; positivity or negativity therefore depending upon the time of flooding.

The second method which they suggest is by pouring plates of nutrient agar incorporated with a bacterial suspension and when cool putting a few drops of the substance to be tested on the surface of the agar. A positive result is indicated by a bacteria free area around the site of the drops. The difficulty here is choosing the proper time at which to test the mold substrate. To say with certainty that a mold produces no substance exhibiting antibacterial activity, samples would have to be taken at repeated intervals over a period of days with the risk, at each sampling, of contamination or of sinking the floating mycelium in the liquid media.

Waksman and Horning (5) likewise use agar plates and their procedures differ essentially from those described above only in the fact that the bacterial organisms are added either before mold inoculation or at a very early stage in the mold

activity, are shown in table 1, experiment 3    Antibiotic activity was exhibited over a wide pH range

#### SUMMARY

A simple method for the qualitative search for antibiotic substances produced by molds is proposed

The results obtained by the use of this method are not dependent upon occlusion of the bacterial culture surface by the molds or upon the pH produced by the molds, and are in all probability a direct measure of the production of antibiotic substances

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# THE ANTISPASMODIC ACTIVITY OF SOME 4-MORPHOLINEALKYL ESTERS<sup>1</sup>

## II RESPONSES OF INTESTINAL MUSCULATURE IN UNANESTHETIZED TRAINED DOGS

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The relaxation produced by some of the morpholino derivatives (8-compounds) in isolated intestinal and uterine segments and in the intestine of anesthetized dogs (1) seemed to warrant further study of the antispasmodic potentialities of this type of compound in unanesthetized subjects. Dogs were chosen for these studies.

### METHOD

Dogs of both sexes were prepared surgically under pentobarbital-ether anesthesia. Operations included Thiry Vella loops, ileostomies and cecostomies. In several instances all three procedures were simultaneously performed in the same animal in order to allow for study of parallel responses of different regions of intestinal musculature to the drugs concerned. Approximately two weeks elapsed for recovery before studies were begun. The dogs were trained to lie quietly with very little restraint and intestinal motility was recorded kymographically by means of the well-known balloon manometric closed system technique (2). Single and double thickness condoms were tied to a catheter in which many holes had previously been cut to allow for ready transfer of air introduced into the closed system. Twenty to thirty cc. of air for ileal registration and from 50 to 120 cc. for colonic recording usually sufficed to produce a desirable degree of distention of the balloons when in place in the intestinal lumen, so that motility changes could be registered from a U tube bromoform manometer (fig. 1). Prior to medication the patterns of normal intestinal activity were established for all dogs included in this series. Control observations endured for periods ranging from two to six hours these intervals conforming to those of medication.

Experiments were first performed after a fast of twelve to eighteen hours but no significant changes in kymographic tracings were noted between fasting and non-fasting subjects and hence no special precautions were usually observed in our later experiments.

At first all drugs, expressed in terms of milligrams per kilogram were ad-

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<sup>2</sup> This section of the project includes the data submitted in thesis form to the Graduate Faculty of Wayne University in partial fulfillment of the requirements for the M.S. degree.

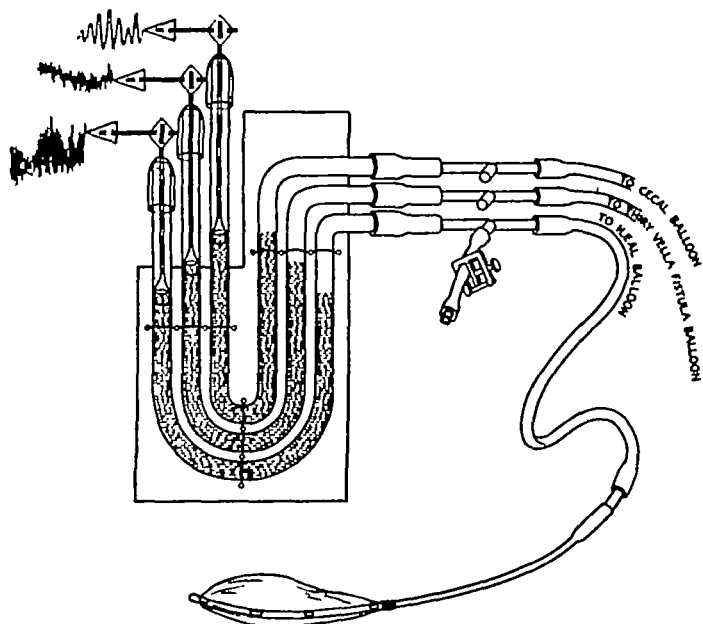


FIG 1

TABLE 1  
S-compounds

SERIES SYMBOL	CHEMICAL NAME	STRUCTURAL FORMULAE	MLD <sub>50</sub> *
S-5	$\beta$ -4-MORPHOLINE ETHYL R R-DIPHENYLACETATE HYDROCHLORIDE		577
S-9	$\gamma$ -4-MORPHOLINE PROPYL R		315
S-14	$\beta$ -4-MORPHOLINE-ETHYL DIPHENYLCHLORO-ACETATE HYDROCHLORIDE		234
S-19	$\beta$ (R-4-MORPHOLINE-ETHOXY-ETHYL-R		208
S-28	$\beta$ , $\beta$ -DIMETHYL- $\gamma$ -4-MORPHOLINE PROPYL R		>2000
S-29	$\beta$ -4-MORPHOLINE-HEXYL R		435
S-35	$\beta$ -4-MORPHOLINE-ETHYL PHENYL CYCLOHEXANE ACETATE HYDROCHLORIDE		594
S-37	$\beta$ , $\beta$ -DIMETHYL- $\gamma$ -4-MORPHOLINE PROPYL PHENYL CYCLOHEXANE ACETATE HYDROCHLORIDE		1500

\* MLD<sub>50</sub> IN 100% KNO<sub>3</sub> INTRAPERITONEALLY IN WHITE MICE. TRASPENTIN 185

mmistered either subcutaneously, intramuscularly or intravenously but for the greater part they were inserted directly into the lumen of the bowel by way of one of the stoma. The latter was accomplished by attaching adjacently a French catheter #10 which delivered solutions at the terminal end of the balloon. The S-compounds (table 1), of which S-14 ( $\beta$ -4-morpholineethyl-chlorodiphenylacetate HCl), S-19 ( $\beta$ -( $\beta$ -4-morpholineethoxy)-ethylidiphenylacetate HCl) S-28 ( $\beta$ - $\beta$ -dimethyl- $\gamma$ -4-morpholinepropyldiphenylacetate HCl) and S-29 ( $\omega$ -4-morpholinehexyldiphenylacetate HCl) appeared to be most promising (1), were administered before and after such drugs as morphine and 1-methyl-4-phenylpiperidine-4-carboxylic acid ethyl ester HCl (Demierol, Winthrop). As our standard for purposes of evaluating spasmolytic potentialities of the new morpholino derivatives,  $\beta$ -diethylaminoethylidiphenylacetate HCl (Trasentin Ciba) was employed.

TABLE 2  
*Lytic effects of S-compounds on morphine spasms*

DRUG	DOSAGE MG./KG I Lp	MORPHINE BEFORE S-COMPOUND		MORPHINE AFTER S-COMPOUND	
		NUMBER OF EXPERIMENTS	RELAXATION OF SPASM	NUMBER OF EXPERIMENTS	PREVENTION OF SPASM
S-29	10	3	3	3	1
	20	1	1		
S-28	10	2	2	1	1
	20	1	1	1	0
	40	1	1	2	2

#### RESULTS

The data reported here were gained from 133 experiments performed on eight dogs and are summarized in tables 2 and 3.

Intravenous injection of the S-compounds rather frequently induced emesis and evidence of general discomfort so that direct insertion of the drug into the bowel loop in doses of 5 to 40 mgm. in 1% or 2% solutions soon became routine procedure. Since the relaxation produced by the S-compounds *intravenously* was transient and was complicated by emetic reactions no valid conclusions as to antispasmodic action could be based upon the variable effects observed. On the other hand *intraloop* administration was always well tolerated without evidence of any type of discomfort and since the gastrointestinal route of administration is that most probable of choice clinically, direct insertion of these drugs into the fistulae seemed appropriate.

Of the S-compounds studied, S-28 and S-29 were most effective in producing some quiescence of intestinal activity after intraloop administration. In some instances tone was depressed more than periodic contractions but frequently the latter were inhibited more than tone. In 24 of 104 experiments or in 23%

normal dog (5a) but usually causes relaxation in the spastic bowel (5b). In several experiments an irritant in the form of Peppermint Water (U.S.P. XII) was inserted directly into intestinal loops which were under the spasmolytic influence of S-28 and S-29. This irritant elicited its usual, anticipated (6) augmentation of intestinal activity.

One of our dogs was hypertensive with a rather uniform arterial tension of 196/102 which persisted for several months after having been treated in a series of dogs with Yohimbine HCl (7). The results obtained from 34 experiments on this dog in this investigation seemed to indicate that there is no difference in response of the intestines of normal and hypertensive animals.

#### DISCUSSION AND COMMENT

The locus of pharmacologic action of the compounds in this series has as yet not been determined. However, since these drugs are effective in isolated tissues (1) as well as *in vivo*, a predominantly peripheral action seems plausible. Whether they have actions similar to those of cocaine, atropine, epinephrine or like those of nitrites and papaverine has not been definitely determined. Since they do not prevent activation of the intestine by such a volatile irritant as Oil of Peppermint they seem to differ from cocaine in that local desensitization of sensory components in the intestinal mucosa is not produced by the S-compounds in the doses employed. It appears that they may resemble atropine or papaverine since they diminish but do not entirely nullify the stimulating action of pituitrin (1) or morphine.

Some structural relationship in chemical configuration would suggest an atropine-like action of these morpholino derivatives (fig. 3). However, previous experiments (1) tend to suggest that a myotropic rather than a neurotropic type of action is characteristic of these compounds. Furthermore, other experiments (8) which are to be reported in detail later, indicate that these agents in intravenous doses of 10 to 40 mgm. have little, if any, anticholinergic action on salivation in urethanized cats and this would seem to preclude a generalized atropine-like action of these S-compounds.

It is also unlikely that the morpholino-derivatives behave like epinephrine since there have been no manifestations of associated epinephrine-like responses such as vascular constriction (1), even after intravenous injection of doses as large as 20 mgm. Arterial tension is momentarily reduced rather than elevated.

The evidence thus seems to favor the concept that these new spasmolytic compounds of morpholino structure approximate nitrites or papaverine in their pharmacologic action on intestinal musculature. Extension of this investigation will include studies with extrinsically denervated Thiry-Vella loops and especially prepared intestinal segments divorced from their intrinsic nerve plexuses (9). It might also be desirable to study cholinesterase and aminoxidase activity within the systems involved.

Clinical appraisal of S-28 and S-29 seems warranted on the basis of results presented and also on the basis of relative toxicity (1). The M.L.D. 50 figures as determined in this laboratory are as follows after intraperitoneal injection

in white mice Trasentin, 185 mgm S-29 435 mgm and S-28 2000 plus mgm. If these toxicity figures prevailed or were of the same order in man the desira

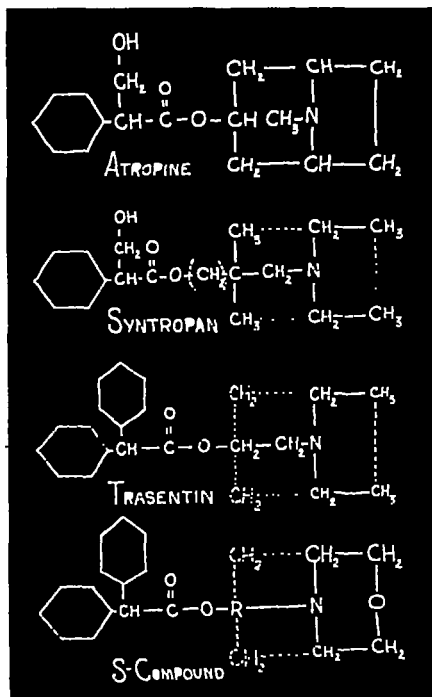


FIG 3 ATROPINE AND ANALOGUES

bility of the latter compounds would be readily apparent. The clinical assay of their spasmolytic activity is now in progress

#### CONCLUSIONS

1 In unanesthetized, trained dogs with ileostomy and colostomy the S-compounds, S-28 ( $\beta$ - $\beta$ -dimethyl  $\gamma$ -4-morpholinepropyldiphenylacetate HCl) and

S-29 ( $\omega$ -4-morpholinhexyldiphenylacetate HCl) are spasmolytic after intraloop insertion in 1% and 2% concentrations in doses of 10 mgm. The degree of spasmolysis is somewhat better than that produced by similar doses of Trasentin.

2 The S-compounds in doses of 20 to 40 mgm. are still more effective, as one would anticipate, than Trasentin in a dose of 10 mgm. Toxicity figures are more favorable for both S-28 and S-29 than for Trasentin and clinical trial seems warranted on this basis of comparison as well as in terms of spasmolytic potency per unit of drug.

3 S-28 and S-29 may prevent the inception of morphine spasm and in well tolerated doses may frequently lyse such spasm.

4 The S-compounds, S-14, S-19, S-28 and S-29, are not too well tolerated by unanesthetized dogs after intravenous administration. This may be due to the transient hypotension produced.

5 The pharmacologic action of these S-compounds on smooth muscle seems to simulate more nearly that of papaverine and nitrites than that of atropine, epinephrine or cocaine.

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# THE ABSORPTION OF 4 4' DIAMIDINO STILBENE (STILBAMIDINE) BY TRYPANOSOMES AND ITS BLOOD CONCENTRATION IN ANIMALS

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4 4' Diamidino stilbene  $(\text{NH}_2(\text{NH})\text{C}_6\text{H}_4\text{CH}=\text{CH}\text{C}_6\text{H}_4\text{C}(\text{NH})\text{NH}_2)$  trade name stilbamidine, has been introduced by Yorke and his colleagues (1) as a powerful chemotherapeutic agent for the treatment of trypanosomiasis leishmaniasis and babesiasis. The present paper describes experiments on the extent of its absorption by trypanosomes in order to investigate the processes by which its trypanocidal action is produced and to compare its action with that of trivalent arsenicals and of acriflavine. Experiments were also made to determine the curve of its blood concentration following injection into laboratory animals.

Stilbamidine exerts a powerful trypanocidal action *in vitro* even in high dilution thus resembling the trivalent arsenicals and acriflavine (i.e. 2 8-diamino-10-methylacridinium chloride hydrochloride) but trypanosomes which have been made resistant to compounds of the diamidine series, e.g. undecanediamidine are not resistant to these latter compounds, and trypanosomes that are arsenical-resistant are not resistant to stilbamidine (2). In its mode of action therefore stilbamidine must seemingly be directed towards a different point of the parasite's external or internal structure. In a previous paper (3) it was shown that trivalent arsenicals and acriflavine are actively absorbed or 'fixed' by the trypanosome: this fixation occurs rapidly (within a few minutes) at 37°C and at 16°C, over a considerable range the concentration of drug inside the trypanosome bears a constant ratio to the concentration in the fluid outside the partition ratio being about 5 000 for reduced trypanasamide and about 8 000 for acriflavine: the fixation is reversible and if the concentration outside the trypanosome be reduced by washing the organisms the concentration inside the trypanosome can similarly be reduced and the first stages of the trypanocidal process can thus be undone. Observations by means of the fluorescent microscope had already shown that stilbamidine is actively absorbed by trypanosomes (4) it was desired to investigate how far the behaviour of stilbamidine resembled the other features of arsenicals and acriflavine which have been described above.

**TECHNIQUE** Stilbamidine exhibits brilliant blue fluorescence when exposed to ultraviolet light and this property can be used for its estimation. The simple method described by Henry and Grindley (5) was employed. Briefly a drop of the biological fluid of which the stilbamidine concentration is to be tested is taken up in a standard dropping pipette and allowed to fall on to a piece of filter paper when dry the spot on the paper is examined under ultraviolet illumination and its fluorescence is compared by visual inspection with that of

drops of the same biological fluid (e.g. serum or urine) containing known concentrations of stilbamidine. In the present work the dropping pipette was held in the hand and two spots were made from each solution so as to minimise individual variation in the area covered by the drop. Comparison of the spots is facilitated by a large condenser to concentrate the rays and suitable filters to reduce the amount of visible light present. Under the conditions of the present experiments, readings could be made over the range 0.8 mgm. to 0.0125 mgm. per 100 cc., concentrations being distinguishable fairly easily from concentrations half or twice as great, intermediate concentrations could be surmised. The spots on the filter paper form stable preparations, although presumably they should be kept dry and in the dark, but in the present work it was found that spots made with a given concentration in one experiment were not always identical with those of the same nominal concentration made during another experiment, so it is preferable to make up fresh standards on each occasion from the solutions employed in the experiment. The brilliancy of the fluorescence was not appreciably affected by moderate changes of the pH of the solution.

The trypanosomes used were *T. equiperdum*, a standard strain maintained in this Institute, and *T. rhodesiense*, the Liverpool strain used by Yorke and his colleagues. The fluid medium consisted of one part of horse serum with three parts of Ringer's solution containing 0.2 per cent glucose.

*Time concentration curves.* Trypanosomes (*T. equiperdum*) were exposed to the action of stilbamidine *in vitro* at 37°C. according to the technique described by Yorke and Murgatroyd (6), and the time required for the various concentrations to kill 90 per cent of the trypanosomes was measured. If the logarithm of the time is plotted against the logarithm of the concentration according to the equation

$$n \log C + \log t = \log k, \quad \text{i.e. } C^n t = k$$

an approximately straight line is obtained ( $C$  = concentration,  $\gamma$  per cc.,  $t$  = time in hours). The figures obtained in the different experiments varied somewhat, but the average values for the constants relating to the rate of action of stilbamidine are

$$n = 0.34, \quad k = 3.2$$

For the trypanocidal action of reduced tryparsamide on *T. rhodesiense*  $n = 0.50$  and  $k = 2.0$ , for that of acriflavine  $n = 0.64$  and  $k = 1.44$  (3).

*Absorption of stilbamidine by trypanosomes.* The general conduct of the experiments was similar to those described in the previous paper (3). A dense suspension of trypanosomes was obtained by differential centrifuging of the citrated blood of a heavily infected rat, and by emulsifying the final deposit of trypanosomes in equal parts of serum and Ringer-glucose. 0.5 cc. of the suspension was mixed with 0.5 cc. of Ringer-glucose containing a suitable concentration of stilbamidine. The tube was incubated at 37°C. for 30 minutes. A drop of the mixture was removed to examine the motility of the trypanosomes and the tube was centrifuged at about 4,000 r.p.m. for 5 minutes. The super-

natant was removed and its stilbamidine concentration was estimated as above. 2.4 cc absolute alcohol containing hydrochloric acid was added to the deposit of trypanosomes which was stirred up; the tube was incubated overnight and then centrifuged; the concentration of stilbamidine in the supernatant was measured in the usual way, the standard spots being made with stilbamidine in acid alcohol.

The results of a typical experiment are given in Table I, which shows that the trypanosomes absorb a large proportion of the stilbamidine initially present. The absorbed stilbamidine can be recovered by extraction of the deposit; the

TABLE I

*The absorption of stilbamidine by trypanosomes (T. rhodensiense) at 37 C. Exposure 30 minutes*

TUBE	CONTENTS	INITIAL CONCENTRATION OF STILBAMIDINE	FINAL CONCENTRATION OF STILBAMIDINE IN SUPERNATANT	CORRECTED CONCENTRATION OF STILBAMIDINE IN EXTRACTED FLUID	PARTITION RATIO
		mgm. per 100	mgm. per 100	mgm. per 100	
1	Living trypanosomes	1.6	0.8		220
2	190,000 per mm <sup>3</sup>	0.8	0.2+	0.8	490
3		0.4	0.1—	0.4	891
4		0.2	0.025+	0.18	1260
5		0.1	0.0125 (approx.)		71600
6	Dead trypanosomes as above	0.2	0.2		
7	Control no trypanosomes	0.4	0.4		
8	Control RBC 50,000 per mm <sup>3</sup>	0.4	0.4		

The trypanosomes in tubes 1-5 were actively motile at the end of the 30 minutes exposure.

The deposit of trypanosomes was extracted with 2.4 cc acid alcohol and the figures in column 5 have been corrected by multiplication by 2.4 to bring back the concentration to that of the original volume viz 1 cc.

figures for the stilbamidine extracted from the trypanosomes plus that remaining in the supernatant tend to total more than the amount initially present, this result being due to the technical error in the estimations. The control tubes 7 and 8 show that no significant amount of stilbamidine is lost by incubation with serum-Ringer glucose alone, or with moderate numbers of red blood corpuscles such as often contaminate the suspension of trypanosomes. Henry and Grindley found that the addition of large numbers of red blood corpuscles (5 million per mm<sup>3</sup> as in undiluted blood) reduced the concentration of stilbamidine in the surrounding fluid by as much as 60-80 per cent, but in my experience small numbers, e.g. 50,000 R.B.C. per mm<sup>3</sup> which are greater than those which

contaminate the suspensions of trypanosomes, do not absorb any appreciable amount of the drug Stilbamidine is not absorbed to a significant extent by dead trypanosomes (killed by heating to 50°C for about 3 minutes), this is contrary to the behaviour of acriflavine or trivalent arsenicals, both of which are avidly absorbed by dead trypanosomes. This small tendency of stilbamidine to be absorbed by dead trypanosomes, taken together with the active motility of the trypanosomes at the end of the period of exposure (Table I), makes it clear that the absorption of stilbamidine is due to the living trypanosomes, it cannot be explained (as is sometimes attempted for acriflavine and arsenicals) by the hypothesis that the drug first kills the cells and that the absorption is merely the non-specific absorption which often occurs with dead tissue. The figures for the partition ratio (concentration inside trypanosomes/concentration outside) is calculated as in the previous paper, the amount of stilbamidine in the trypanosomes is given by the amount which has disappeared from the supernatant, e.g. 0.8 mgm per 100 cc in Tube 1, i.e. 8  $\gamma$  in 1 cc, the volume of the trypanosomes is obtained from the number given, the volume of 100 million trypanosomes being taken as 2.4 mm<sup>3</sup> (experimental determination). It is seen from Table I that the partition ratio tends to diminish as higher concentrations of stilbamidine are present.

The relation between the concentration of stilbamidine outside the trypanosomes and that inside for a number of experiments is represented graphically in Fig. 1. There is a considerable variation between the different results, particularly in the experiments with *T. equiperdum*. In the previous work with acriflavine (3) it was found that the curve, plotted as logarithms, formed a straight line at an angle of 45° for a considerable part of its course, flattening off with the higher external concentrations, i.e. the partition ratio was constant over a considerable range and the flattening with the higher concentrations was interpreted as a (hypothetical) saturation of the trypanosomes' receptors for the drug. In the present experiments, although there is a tendency for the curve to flatten off in the higher concentrations, the slope in the lower concentrations is seldom as steep as 45°. The experimental variation is too great to decide whether the partition ratio in this region should really have a constant value or not, and the question did not seem to have sufficient importance to justify further work on the subject. Provisionally the geometrical mean was taken for experimental results obtained when the external concentration was in the range 0.01–0.001 mgm per 100 cc. The mean figure for the partition ratio with *T. rhodesiense* (10 readings) is accordingly 1100 (170–1980) and that for *T. equiperdum* (9 readings) is 1700 (340–31,000), the difference between the figures for the two species of trypanosome has no significance. The partition ratio for reduced tryparsamide is about 5,000 and that for acriflavine is about 8,000 (3), so that apparently stilbamidine is absorbed less avidly than these two compounds.

**Rate of absorption** The rate of absorption of stilbamidine was investigated by suspending trypanosomes in suitable concentrations of the compound, after various intervals the tubes were centrifuged (during which process the trypanosomes were in effective contact with the drug for 2–4 minutes) and the super-

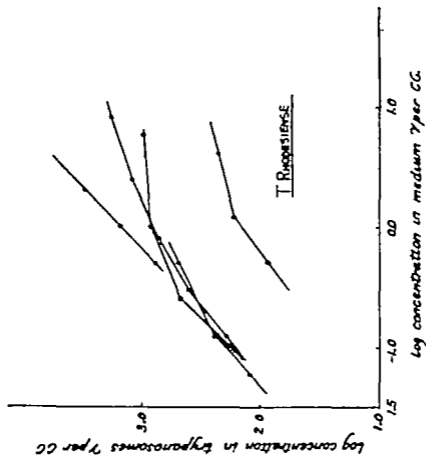
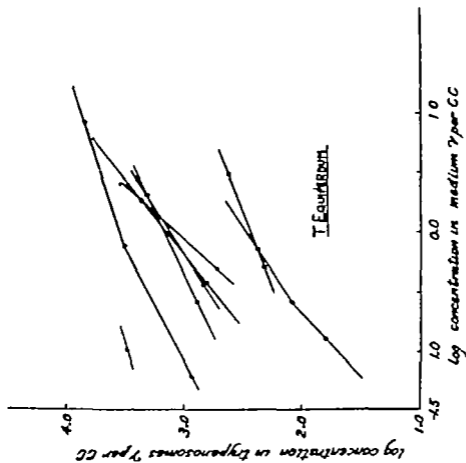


FIG. 1 The relation between the concentration of stilbamidine in the medium and the concentration in the trypanosomes at 37°C after equilibrium has been reached (30 minutes exposure)

natant removed, its stilbamidine content being estimated in the usual way. The rate of absorption varied considerably in the different experiments, but a typical result is shown in Table II. On this occasion at 37°C 87 per cent of the compound was absorbed in 10 minutes plus the period of centrifuging, usually a period of 5-13 minutes was required for half the compound to be absorbed. At room temperature measurable absorption rarely occurred even in 30-45 minutes. With acriflavine, absorption is rapid both at 37°C and 16°C, 1-5 minutes sufficing for most of the drug to be removed (7), with reduced tryparsamide, absorption is similarly rapid (8). Stilbamidine is absorbed more slowly than either of these two compounds, the negligible absorption which occurs at 18°C being especially remarkable.

*Reversibility of absorption.* The attempt was first made to demonstrate the reversibility of absorption by the direct method of showing that the stilbamidine

TABLE II

*The rate of absorption of stilbamidine by trypanosomes. Initial concentration of stilbamidine, 0.4 mgm per 100 cc*

Number of trypanosomes (*T. equiperdum*) 50,000 per mm<sup>3</sup>

TUBE	TEMPERATURE	TIME OF EXPOSURE	CONCENTRATION OF STILBAMIDINE IN SUPERNATANT	PERCENTAGE OF STILBAMIDINE ABSORBED
	C	minutes	mgm per 100 cc	
1	37	1	0.4	0
2		5	0.15	37
3		10	0.05	87
4		30	0.0125 or less	97
5	18	10	0.4	0
6		25	0.4	0
7		45	0.4	0

content of trypanosomes was diminished by washing them. A heavy suspension of trypanosomes was taken and exposed *in vitro* to stilbamidine. The suspension was divided into several parts, with one portion the trypanosomes were separated by centrifuging at once and their stilbamidine content was estimated after extraction with acid alcohol, in the other portions, the trypanosomes were washed several times and their stilbamidine content was estimated. No significant diminution of stilbamidine content could be demonstrated after washing three times. However, calculations based on the partition ratio showed that the volume of washing fluid, which was practical to handle, was too small for any significant removal to be expected. Accordingly the reversibility of absorption was investigated by an indirect method depending on the conception that, if the combination between drug and trypanosome is reversible, the presence of unabsorbed drug in the medium will be necessary to prevent the drug diffusing back out of the trypanosome again.

*Experiment.* A series of parallel tubes were set up containing trypanosomes, 1,500 per mm<sup>3</sup> and stilbamidine, 0.4 mgm per 100 cc. These were incubated at 37°C for various

periods and then centrifuged the deposit of trypanosomes was washed once with dilute serum and enough fresh medium was added to bring the volume back to its original dimensions. The survival of the trypanosomes was followed by making frequent counts and the findings were plotted as time action curves. Fig. 2 shows the curve for selected tubes viz. the tube with unwashed trypanosomes, a tube with trypanosomes washed after 20 minutes and a control tube without drug treated in the same way. The number of organisms in the tubes is expressed as a percentage of the number found after washing and adding fresh medium.

These curves show that the trypanosomes left in contact with the drug are almost all dead in  $2\frac{1}{2}$  hours but that most of those which had been exposed for 20 minutes (during which period they had time to absorb the compound and to

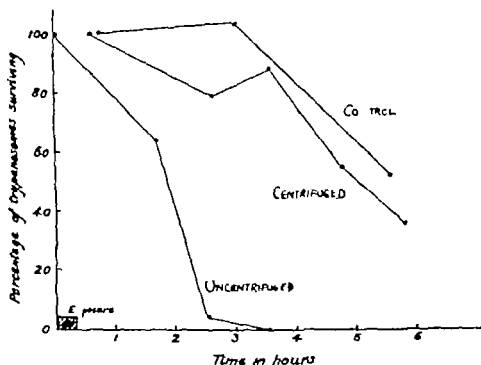


FIG. 2. Showing the survival of trypanosomes after exposure to stilbamidine if centrifuged off, washed and suspended in fresh medium. Concentration of stilbamidine 0.4 mgm per 100 cc. Duration of exposure, 20 minutes. Temperature  $37^{\circ}\text{C}$ . Initial number of trypanosomes 1,500 per mm<sup>3</sup>.

come into approximate equilibrium with the environment) and then washed lived almost as well as the trypanosomes in the control tubes. It is concluded that the drug which had been fixed by the trypanosomes in the first part of the experiment has been washed out again during the second part and that the fixation of stilbamidine is reversible as is that of trivalent arsenicals and acriflavine. However the reversibility of fixation is less easy to demonstrate for stilbamidine than it is for reduced trypanamide and for acriflavine and probably it occurs less readily with stilbamidine than with the two latter compounds.

*Absorption of stilbamidine by trypanosomes in vivo.* This has already been demonstrated by Hawking and Smiles (4) who showed that when an infected mouse was treated with stilbamidine and a thin film of the blood was spread on a glass slide the trypanosomes were brightly fluorescent if examined with ultra violet light. Further proof of the absorption *in vivo* was obtained in the

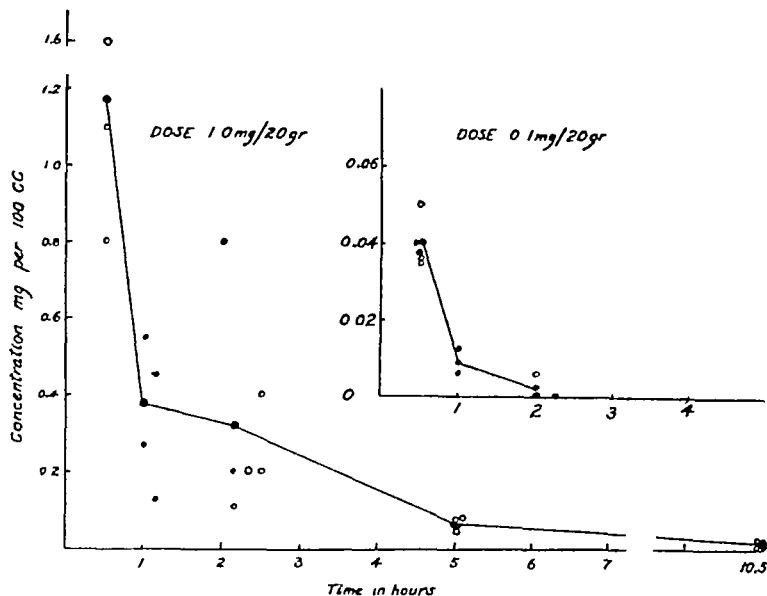


FIG 3 The plasma concentration of stilbamidine in mice following intraperitoneal injection of the compound. The circles indicate the concentrations in individual mice, the dots, joined by the line, indicate the average concentrations of the groups.

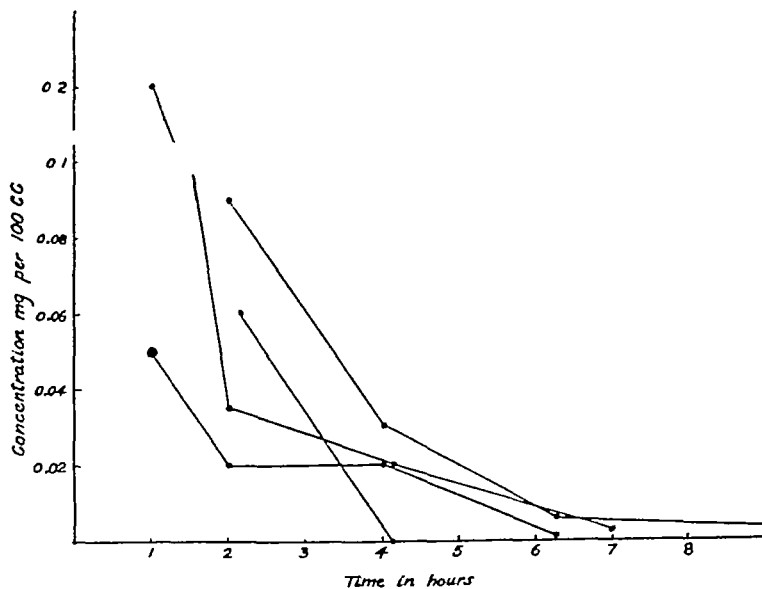


FIG 4 The plasma concentration of stilbamidine in rabbits following intravenous injection of 10 mgm per kgm. Each line represents a different animal.

present experiments. Heavily infected rats were given stilbamidine 2.5 mgm per kgm, by intraperitoneal injection, and they were killed after 15 minutes. The blood was collected over citrate and the trypanosomes were separated off and extracted with acid alcohol. From  $4.5 \times 10^8$  trypanosomes, 0.12 mgm stilbamidine was recovered. The concentration of stilbamidine in the plasma was 0.25 mgm. per 100 cc. so that the partition ratio was 4,400 but it is not certain whether equilibrium had been reached. This recovery of stilbamidine from the trypanosomes of a treated animal is similar (in proportion to the dose) to that obtained with acriflavine (7) and with reduced tryparsamide (8).

*Plasma concentration of stilbamidine in rabbits and mice.* The plasma concentration of stilbamidine following intravenous (rabbits) or intraperitoneal (mice) injection of the compound was studied by collecting the blood over citrate, centrifuging and estimating in the usual way. In rabbits great difficulty was encountered in obtaining the earlier samples of blood since the drug caused intense vaso-constriction which persisted for 3-4 hours in mice this difficulty did not occur as the mice were killed and blood was collected from the heart. The results of these experiments are represented in Figs. 3 and 4. Assuming that a 1 kgm rabbit contains 62 cc blood, the theoretical concentration in the blood after injection of 10 mgm stilbamidine would be 16 mgm per 100 cc. Similarly in mice assuming 1.2 cc blood per 20 grams the maximum concentration in the blood following the injection of 10 mgm. would be 80 mgm. per 100 cc or, if the compound were evenly distributed over the whole animal the concentration would be 5 mgm per 100 cc. From the data of Figs 3 and 4 it is clear that stilbamidine quickly disappears from the plasma, even during the first half hour after administration.

**DISCUSSION** According to Clark (9) "the addition of a drug to a population of cells causes the following events: 1) fixation of the drug by cells; 2) secondary chemical reactions between the drug and cell constituents; and 3) biological response to injury—in this case, death of the trypanosomes. The experiments described above deal mostly with the first phase viz fixation. As has been shown the fixation of stilbamidine by trypanosomes occurs moderately quickly; there is a quantitative relation at equilibrium between the concentration inside the cell and that outside (partition ratio over the range of concentrations studied is approximately 1,400) and the fixation is (possibly incompletely) reversible. In these and other respects the behavior of stilbamidine towards trypanosomes closely resembles that of trivalent arsenicals and probably it will ultimately be found that the mode of action of all these three compounds is of a similar type. On the other hand as was mentioned in the introduction, the non identity of drug resistance to arsenicals and of that to diamidine compounds indicates that stilbamidine is fixed at a different point on the trypanosome to the one at which arsenicals and acriflavine are fixed. Also the fixation of stilbamidine occurs more slowly than that of the other two compounds and it is probably reversed less readily.

From the quantitative data given above various calculations may be made about the trypanocidal action of stilbamidine. Thus since  $C_{ant} = 3.2$ , a con-

centration of stilbamidine, 1.9  $\gamma$  per ml kills trypanosomes (1,500 per mm<sup>3</sup>) in about 4 hours. The partition ratio for *T. equiperdum* is 1,700. Accordingly, the quantity of stilbamidine absorbed by the trypanosomes is 0.073  $\gamma$  per million trypanosomes. Therefore, the amount required to kill a trypanosome in 4 hours is about  $7.3 \times 10^{-8} \gamma$ . Assuming that 1 gm mol contains  $6.4 \times 10^{23}$  molecules, this would correspond to  $1.8 \times 10^8$  molecules per trypanosome. The corresponding quantities for acriflavine are  $9.2 \times 10^{-8} \gamma$ , i.e.  $2.7 \times 10^8$  molecules, and for reduced tryparsamide  $7.8 \times 10^{-9} \gamma$ , i.e.  $1.1 \times 10^7$  molecules per trypanosome (3). Assuming that the area covered by an adsorbed molecule of stilbamidine is about the same as that covered by an adsorbed molecule of methylene blue, viz  $5 \times 10^{-7}$  sq ( $\mu$ ), and that the surface area of a trypanosome, measured as a cylinder 25  $\mu$  long and 1.5  $\mu$  in diameter is about 120 sq  $\mu$ , it may be calculated that the quantity of stilbamidine required to kill a trypanosome would be enough to cover 90 sq  $\mu$  if spread out as a mono-molecular film, i.e. most of the area of the trypanosome. However, examination by the fluorescent microscope of trypanosomes which have absorbed stilbamidine shows that the compound is *not* evenly distributed over the surface, but that it is concentrated into the blepharoplast and certain other granules, the rest of the cytoplasm and the nucleus take up little or no fluorescent material (4).

Consideration may be given to the question how far the behaviour of stilbamidine towards the trypanosome and towards the animal host may be correlated with its electro-chemical properties. Stilbamidine as the salt of a strong base is highly ionized in dilute solution (5) and its activity might be attributed to its kationic nature. It has, however, been suggested (10) that the trypanocidal activity of long-chain amidines, guanidines and isothiourreas is a property of the free bases liberated by hydrolysis. Acriflavine is the salt of a strong quaternary base which cannot act in solution except as a strong kation. Reduced tryparsamide ( $\text{NH}_2 \cdot \text{CO} \cdot \text{CH}_2 \cdot \text{NH} \cdot \text{C}_6\text{H}_4 \cdot \text{As} (\text{S} \cdot \text{CH}_2 \cdot \text{COONa})_2$ ), which resembles these so closely in its behaviour towards trypanosomes, is the sodium salt of a weak acid, and in solution would function as an anion. There is, however, considerable evidence (11) that in solution hydrolysis occurs at the arsenic-sulphur linkage, with the production of the arsenoxide- $\text{NH}_2 \cdot \text{CO} \cdot \text{CH}_2 \cdot \text{NH} \cdot \text{C}_6\text{H}_4 \cdot \text{As} = \text{O}$ . Such a molecule is weakly amphoteric, carrying as it does a weakly negative group ( $-\text{AsO}$ ) and a weakly positive group ( $-\text{NH}-$ ). Accordingly, the action of this particular group of compounds cannot easily be fitted into any scheme of anionic and kationic types of antiseptics, which have been discussed by Albert (12). The behaviour of stilbamidine and of acriflavine towards the host, viz high toxicity and rapid disappearance from the blood stream, is typical of electro-positive substances, on the other hand, reduced tryparsamide behaves similarly in this respect in spite of its weak electronegative charge, so that much significance cannot be attached to this fact.

#### SUMMARY

The rate of trypanocidal action of stilbamidine *in vitro* for *T. equiperdum*, under the conditions described in the paper, is in approximate agreement with the equation

$$(\text{Concentration})^{0.24} \times \text{time} = \text{constant}$$

Stilbamidine is absorbed by trypanosomes *in vitro* with moderate rapidity. When equilibrium is reached there is a quantitative relation between the concentration inside the trypanosome and that outside (over the range of concentrations studied the concentration inside is about 1,400 times as great as the concentration outside). Absorption during its early stages is reversible. Absorption of stilbamidine by trypanosomes also occurs *in vivo*.

It is calculated that the amount of stilbamidine required under these experimental conditions to kill a trypanosome in 4 hours at 37°C is  $7.3 \times 10^{-8}$  molecules.

The behaviour of stilbamidine towards trypanosomes is closely similar to that of trivalent arsenicals and of acriflavine although the facts about drug resistance (2) indicate that stilbamidine is absorbed by a different part of the parasite from that which absorbs these other compounds.

Curves are given showing the concentration of stilbamidine in the plasma of rabbits and mice following intravenous or intraperitoneal administration. The compound rapidly disappears from the plasma. Sufficient stilbamidine was injected intravenously into rabbits to yield a (theoretical) concentration of 16 mgm. per 100 cc. after two hours the actual concentration was about 0.05 mgm. per 100 cc. and after six hours it was less than 0.005 mgm. per 100 cc.

Grateful acknowledgments are due to Miss R. J. Berson for technical assistance.

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# SOME CIRCULATORY EFFECTS OF PRIVINE HYDROCHLORIDE

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2-Naphthyl-1'-methylimidazoline HCl has been introduced as a potent topical vasoconstrictor under the trade name of privine, in buffered 0.1 and 0.05% solutions for use as a nasal decongestant. Crystalline privine hydrochloride was supplied by the Ciba Pharmaceutical Co., Inc., of Summit, N. J., for the present study.

Privine is of interest since it is unrelated chemically to the sympathomimetic amines, yet shows high activity as a vasoconstrictor. Its local effects have been studied by several workers (1-3). No systemic toxic effects were noted (4) in 104 patients in whom a 0.1% solution was applied to the nasal mucosa, or after instillation of this solution into the sinuses. Yonkman (5) similarly found little systemic effect in the dog after introduction of massive doses into the nasal cavity or intestine. However, Meier and Müller (1) note undesirable actions of 5 allied compounds. Of these related agents, phenylamino-methylimidazoline has been shown (6) to cause cardiac irregularities and diminished minute volume in the rabbit heart-lung preparation. Further study of systemic actions of privine is indicated to evaluate properly the possible hazards in the clinical application of privine.

Dogs were given 30 mg/kg Na pentobarbital intraperitoneally and supplemental Na barbital intravenously as needed. Kymograph records were made of carotid pressure and respiration. In some experiments, a sensitive tambour was also connected to the carotid artery to magnify changes in pulse pressure.

Pressor responses to intravenous injections of privine vary in different dogs. Although the lowest certain pressor dose lies between  $1-2 \times 10^{-5}$  mM/kg, the response to  $2 \times 10^{-5}$  mM/kg varies from 2-35 mm Hg with a mean of  $20.4 \pm 12.5$ . Larger doses do not cause a proportionate increase in response. Doses  $> 5 \times 10^{-5}$  mM/kg frequently produce cardiac irregularities which limit the pressor response. A rise of 45-60 mm Hg is usually found with this dose, but occasionally the response is less than that with  $\frac{1}{2}$  this dose. With  $10^{-4}$  to  $10^{-3}$  mM/kg, privine frequently evokes only a slight pressor effect.

Significant tachyphylaxis appears with doses of  $5 \times 10^{-5}$  mM/kg. It is usually not appreciable with lower doses, although a dog treated with repeated injections of  $4 \times 10^{-5}$  mM/kg at 30 min intervals showed 7 consecutive pressor responses of 40, 22, 16, 12, 14 and 10 mm Hg. Neosynephrine in the same dose, given similarly, yielded consecutive responses of 42, 38, 44 and 46 mm Hg while phenylpropylmethylamine HCl (vonedrine), in  $50\times$  this dose, produced rises of 84, 70, 74, 54 and 48 mm Hg. Yonkman (5) has noted that pentobarbitalized dogs are particularly prone to show tachyphylaxis with privine, but the phenomenon has been noted in dogs 8-10 hours after pento-



FIG. 1 Effects in the pentobarbitalized atropinized dog of some pressor amines and privity on carotid pressures, pulse pressure and respiration. All doses are expressed in  $\text{mg}/\text{kg}$ . At A  $5 \times 10^{-4}$  epinephrine B  $5 \times 10^{-4}$  neophrine C  $D$  E and F,  $2 \times 10^{-4}$  H  $10^{-4}$  2  $10^{-4}$  and  $4 \times 10^{-4}$  privity resp G  $3 \times 10^{-4}$  phenpropylmethylamine (vonedrine) H  $3 \times 10^{-4}$  amphetamine and I  $2 \times 10^{-4}$  ephedrine

barbital was given, Na barbital was administered to these dogs to maintain narcosis

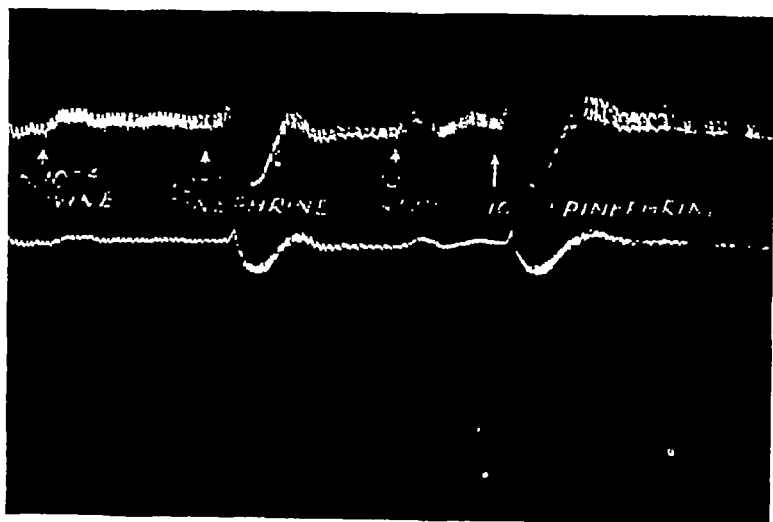


FIG 2 DEPRESSOR RESPONSES TO EPINEPHRINE FOLLOWING LARGE DOSES OF PRIVINE

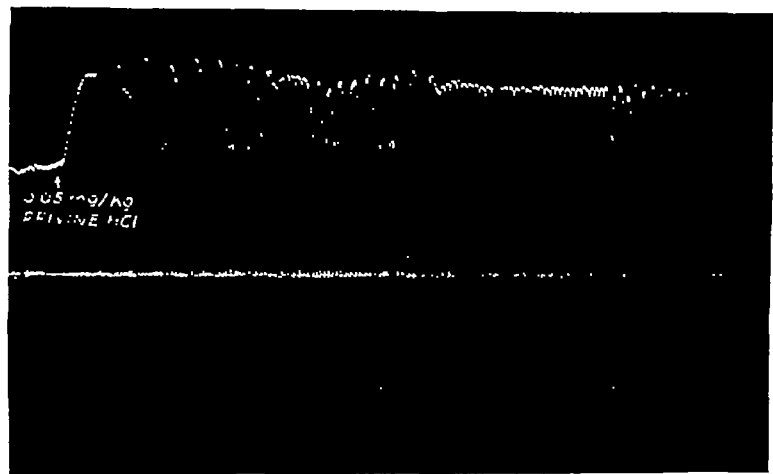


FIG 3 CARDIAC IRREGULARITIES WITH PRIVINE IN THE ATROPINIZED DOG

Atropine sulfate, 1-2 mg/kg intravenously, does not influence the response to moderate doses of privine. Cocaine HCl, 10 mg/kg subc, does not potentiate the response. Yonkman (5) states that a slight potentiation occasion-

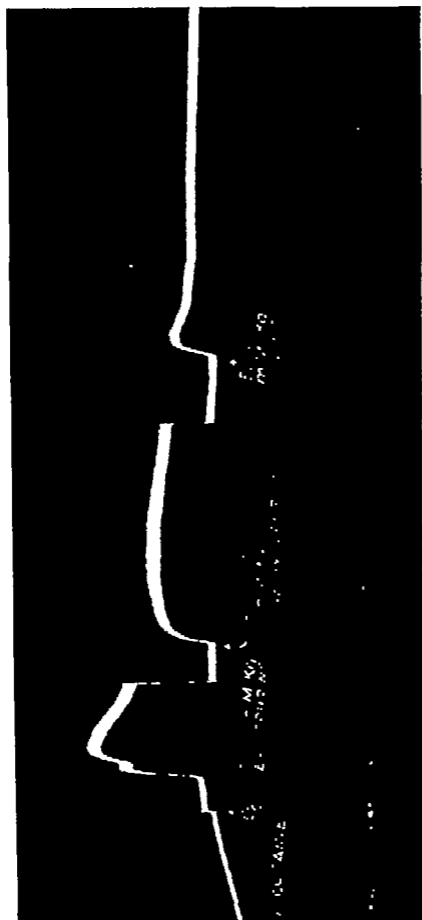


FIG 4 EFFECTS OF EPINEPHRINE NEOSYNEPHRINE AND PRIVINE IN THE COCAINATED ATROPINIZED DOG

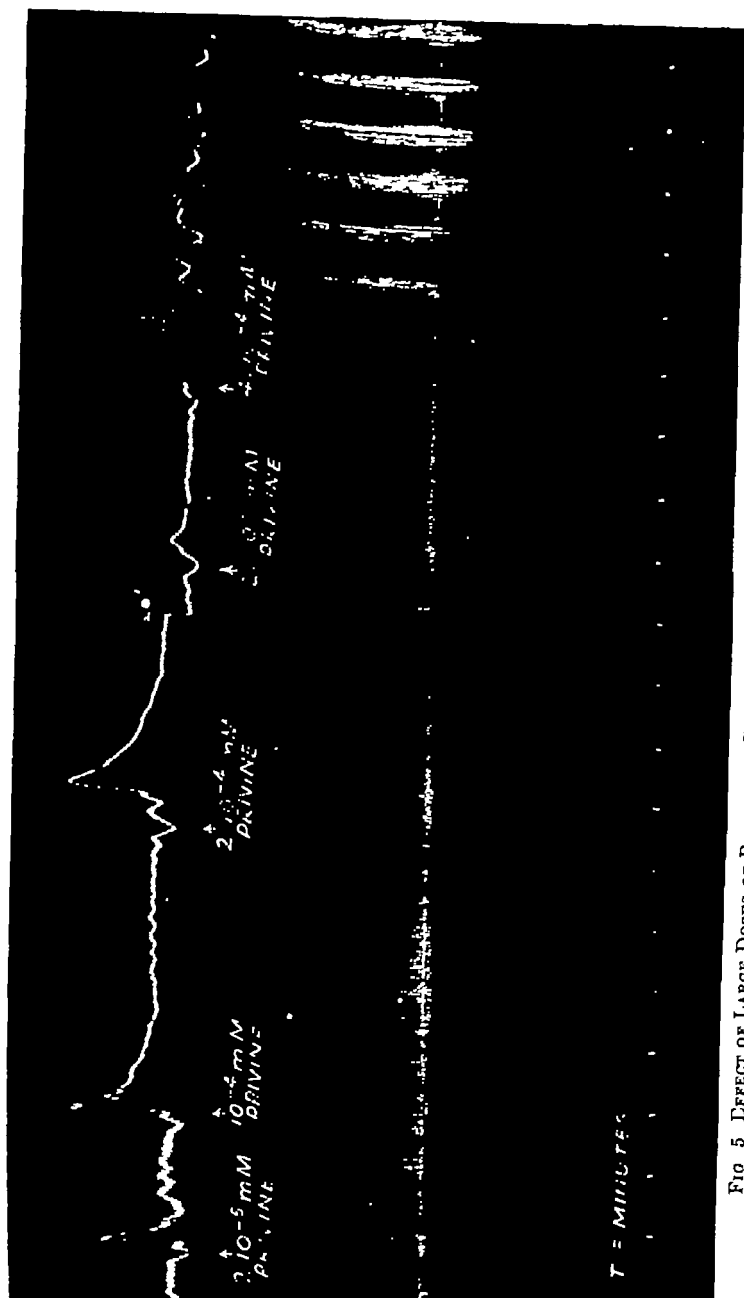


FIG 5 EFFECT OF LARGE DOSES OF PRIVINE ON CAROTID PRESSURE AND RESPIRATION IN THE ATROPINIZED DOG

ally occurs, but the type of narcosis used may govern this effect. Yohimbine and ethilyohimbine are said (5) to decrease but not reverse the pressor action of prinine. In the present study, piperidinomethylbenzodioxane 933F, was never found to reverse the action of prinine when given in adrenolytic doses of 1 mg/kg or sympatholytic doses of 10 mg/kg, but reduced it in either case. Further, the depressor response to subsequent injections of epinephrine was greater by some 50% following prinine. This apparent enhancement of the adrenolytic action of 933F by prinine suggested that prinine itself might be adrenolytic in proper dose although small doses potentiate epinephrine, as noted by Yonkman (5). A dog which tolerated large doses of prinine without cardiac irregularities showed a strong adrenolytic action of prinine.

Certain of these findings may be conveniently illustrated. Figure 1 presents a comparison of pressor effects of prinine and some sympathomimetic amines and demonstrates potentiation of epinephrine by prinine. Figure 2 illustrates depressor responses to epinephrine following large doses of prinine. This record followed a series of alternate injections of epinephrine and increasing doses of prinine, an initial rise of 64 mm Hg following  $2 \times 10^{-4}$  mM/kg of epinephrine was thereby converted into the fall of 60 mm Hg after  $10^{-4}$  mM/kg of epinephrine, as noted in figure 2. The action of cocaine in potentiating epinephrine strongly and neosynephrine less strongly is noted in figure 4 the response to prinine after cocaine is slightly less than previous responses before and after atropinization. In figure 3, cardiac irregularities after injection of 0.05 mg/kg of prinine HCl are noted, and figure 5 shows responses to doses of prinine increasing from  $2 \times 10^{-4}$  to  $4 \times 10^{-4}$  mM/kg. Tachyphylaxis is noted on the 2nd injection of  $2 \times 10^{-4}$  mM/kg. The terminal Cheynes-Stokes' respiration was frequently encountered when large doses of prinine were given.

#### SUMMARY

Circulatory effects of prinine HCl on intravenous injection are compared with those of some sympathomimetic amines. In dogs narcotized with pentobarbital the minimal pressor dose is  $1-2 \times 10^{-4}$  mM/kg while a maximum response is usually obtained with  $5 \times 10^{-4}$  to  $10^{-4}$  mM/kg. Above this dose, prinine generally causes cardiac irregularities which limit the pressor response. Tachyphylaxis occurs at about this dose. Atropine and cocaine do not appreciably influence the action of prinine. 933F reduces but does not reverse the pressor action. Small doses of prinine potentiate epinephrine but large doses are adrenolytic. Cheynes-Stokes' respiration often follows injection of large doses.

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# THE INFLUENCE OF "HEART STIMULANTS" ON THE CONTRACTION OF ISOLATED MAMMALIAN CARDIAC MUSCLE<sup>1</sup>

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This study was undertaken because of 1) recurring statements in the literature and in pharmacology text-books that certain substances stimulate the myocardium directly to increase its force of contraction after therapeutic doses, 2) the lack of conclusive supporting evidence and 3) the availability of a method by which direct evidence might be obtained. The substances investigated were those most commonly designated as, and used as, "circulatory" or "cardiac stimulants" camphor, the so-called camphor substitutes pentamethylene tetrazol ("Metrazol" or "Cardiazol") and nikethamide (the diethyl amide of nicotinic acid ["Coramine"]), the xanthines caffeine, theobromine and theophylline, barium chloride, and epinephrine and ephedrine.

Most previous work bearing on this problem has been carried out either on intact animals or on isolated heart or heart-lung preparations. Obviously, the several factors governing the force of the heart in situ, and even in the isolated heart preparations, cannot be completely controlled and consequently the peripheral vascular, nervous, and coronary actions of these several drugs could not be separated from any direct myocardial action which they may possess. The isolated papillary muscle method of Cattell and Gold (1) permits elimination of these uncertainties in the interpretation of data bearing on drug action directly on mammalian heart muscle.

**METHOD** The test object used in this study was the papillary muscle of the right ventricle of the cat heart. The preparation was the same as that used in demonstrating the action of digitalis glycosides in augmenting the force of failing heart muscle (1). The muscles were usually used soon after excision, but sometimes they were stored in the refrigerator in Locke's solution 3 to 24 hours before use. The muscle preparations were made to contract by electrical stimulation at a regular rate of 35 to 40 per minute at constant resting or "diastolic" tension. Photographs of the tension response were taken at intervals determined by rate of change and were plotted against time. When the contractile force had become stabilized at a given level or was falling but slightly, the drug was added in an amount sufficient to give the desired concentration in the Locke's solution without uncovering the muscle. It should be emphasized that varying degrees of failure were present under these conditions and therefore provided favorable conditions for the demonstration of stimulant action. To test the responsiveness

<sup>1</sup> Taken in part from a dissertation presented to the Graduate School of Cornell University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in September 1942.

of a muscle preparation to an adequate stimulus, epinephrine in 1:1 million or 1:10 million was applied terminally.

An effort was made to relate the concentration of a drug resulting in an increase of 'systolic' tension to the concentration estimated to be present in man following therapeutic doses. This comparison is of interest in judging whether a drug as employed in therapeutics may be expected to be effective in stimulating the myocardium.

I CAMPHOR, PENTAMETHYLENE TETRAZOL<sup>1</sup> AND NIKETHAMIDE.<sup>2</sup> The belief that camphor and its so-called substitutes pentamethylene tetrazol and nikethamide stimulates the mammalian myocardium to increase its force of contraction rests on the results of experiments carried out on isolated hearts and heart lung preparations (2, 3, 4, 5, 7) the test objects either were in spontaneous failure or were poisoned by such substances as chloral chloroform muscarine, etc. camphor then being added. In many cases the concentrations of camphor used were very high. Similarly *in vivo* experiments led to similar conclusions (4, 5, 6, 7). However, contrary results and interpretations have also appeared (8, 9, 10, 11).

Pentamethylene tetrazol and nikethamide were considered the most promising of a group of so-called 'soluble camphor' compounds. As in the case of camphor cold and warm blooded hearts have served as test-objects often poisoned by substances of great diversity of action, such as chloroform and strophanthin. Increased rate and amplitude and initiation of activity when it has ceased have been reported (8, 12, 13, 14, 15, 16, 17). Contrariwise many have reported no stimulating action either in isolated preparations or *in situ* even in very high concentrations (18, 19, 20, 21, 22, 23, 24, 25).

Nikethamide has been stated to augment the frequency and amplitude of cold and warm blooded hearts even when these have been poisoned with various agents such as chloral chloroform choline, etc., and by calcium lack (26, 27). It has been stated that ineffective doses of digitalis or strophanthin may be rendered active by nikethamide (28, 29), and that the action of nikethamide resembled that of digitalis (26). On the other hand, many investigators have been unable to observe such actions (15, 18, 19, 20, 21, 30) in hearts either in the normal or failing state isolated or *in situ*.

The present study of the action of these compounds on isolated mammalian cardiac muscle was undertaken in an effort to determine whether stimulation (direct) of mammalian heart muscle is possible in any concentration with the hope of shedding some light thereby on the conflicting experimental results of past years. A preliminary report has appeared elsewhere (31).

*Results Camphor* From twelve experiments in each of which one to three observations were made the following results were obtained. Camphor in concentrations of from 1:1 million to 1:25,000 was without effect on the performance of the preparations. Concentrations greater than 1:25,000 i.e., 1:20,000 to 1:2,000 caused progressive depression according to concentration, and concen-

<sup>1</sup> 'Metrazol' or 'Cardiazol'

<sup>2</sup> Pyridine  $\beta$ -carboxylic acid diethylamide or 'Coramine'

trations of 1 2,000 almost invariably caused irreversible inexcitability very rapidly (2 to 5 minutes) In these latter concentrations, varying degrees of contracture occurred in most instances concomitantly with the diminution of "systolic" tension If the toxic action of the higher concentrations was not permitted to proceed too long, it could be partially reversed by unmodified Locke's solution At the higher camphor concentrations, in which alcohol was necessary to obtain solution, control studies were carried out of the effect of alcohol at corresponding concentrations It was thus established that the alcohol alone accounted for from 25 to 50 per cent of the depression In one preparation in which survival was particularly good, the concentration of alcohol was maintained constant—(0.2 per cent)—and the camphor concentration was varied, fresh Locke's solution being substituted between camphor applications The degree of depression bore a rough relation to the camphor concentration

*Comment* The action of camphor appears to be solely depressant The findings reported here lead to an interpretation at variance with that of the older investigators who observed apparent "stimulation" of the myocardium with camphor This discrepancy can probably be largely explained on the basis of Loewi's observation that camphor decreases the cardiac effects of electrical stimulation of the vagus nerve It has recently been confirmed that chloroform, chloral hydrate, ether, and other depressants (32) inhibit cholinesterase in suitable concentrations, thus camphor may block the inhibitory effects of acetylcholine or muscarine, thereby increasing the rate and (indirectly) the "amplitude" of the response It is noteworthy that in many instances in which camphor "antagonism" to other drugs has been reported, the force of the heart was not affected, and it is possible that the muscular effects of the poisons were not reversed but actually enhanced by camphor

*Pentamethylene tetrazol* Most of the experiments with pentamethylene tetrazol were performed about a concentration range of 1 17,000, inasmuch as this is the concentration attained in the blood of a man of average size after the intravenous dose, 0.1 to 0.3 gram, recommended by "New and Nonofficial Remedies"—a few were performed in the range of 1 1000 to 1 5000 and a few at 1 50,000 and 1 100,000 From eleven experiments in each of which one to four observations were made, the following results were obtained

Even in the highest concentrations employed (1 1,000) pentamethylene tetrazol was ineffective in raising the "systolic" tension of the papillary muscle preparation Upon replacing the drug with unmodified Locke's solution, no changes in tension (other than the slight changes incidental to changing the solution) occurred in the majority of experiments, indicating lack of toxicity even in high concentrations In 2 out of 8 experiments, some recovery occurred with such treatment, but these two preparations had previously been exposed to moderately high concentrations of nikethamide and therefore the result may have been due to a residual quantity of that drug As further evidence of low toxicity, there was an insignificant change in the resting tension (expression of tendency to change length) even after concentrations as high as 1 2,000 Excitability to electrical stimulation likewise was unmodified

*Comment* The results are in agreement with the findings of Camp (22), Leyko (18) Barker and Levine (23) Messinger and Moros (25) and others. Its action on other structures must therefore account for any cardiac changes in the intact animal

*Nikethamide* The majority of the experiments with nikethamide were performed in the range of concentrations obtaining in the circulation of man after intravenous injection of a large dose e.g. 0.75 gram thereby giving concentrations approximating 1:7,000, and a few experiments using lower and higher concentrations. Concentrations as high as 1:10,000 were without effect. In 1 experiment out of 7 depression was noted after 1:10,000 but in the majority of experiments depression was not seen except in concentrations of 1:2,000 or higher. The depression was not marked although recovery did not take place while the muscle remained immersed in the drug solution. Sometimes such concentrations increased the resting tension somewhat. Replacement by unmodified Locke's solution usually resulted in prompt resumption of the control response and sometimes this was slightly exceeded. Concentrations of nikethamide between 1:10,000 and 1:200,000 were without significant effect.

*Comment* Nikethamide does not stimulate the mammalian myocardium by a direct, primary action. The results of Leyko (18) and others (33) indicated that coronary dilatation takes place in the mammalian heart-lung preparation and this may account for some of the results of other workers who observed an increase in amplitude, since hypodynamic preparations might be influenced by coronary changes. The results of this report agree with those of Leyko and of Peters and Visscher (30) in demonstrating that the working capacity of cardiac muscle is not augmented by nikethamide. Reports of clinical success must therefore be regarded with caution and interpretation must be tempered with consideration of the other actions of nikethamide.

*II BARIUM CHLORIDE.* Most textbooks of pharmacology state that barium chloride is capable of increasing the irritability of heart muscle to the extent of preventing attacks of syncope in complete auriculoventricular dissociation (Stokes-Adams Syndrome). This appears to be well supported by clinical experience (34 and others). Further the statement is made that barium has a digitalis-like action in increasing the force of the heart beat. Rothberger and Winterberg (35) and van Egmond (36) found that in the normal dog's heart and in the dog's heart with complete block, 5 to 100 mg. per kg. of barium chloride caused increased ventricular excitability evident sometimes as extra systoles sometimes as regular idioventricular rhythms of varying degrees of persistence. In addition increased amplitude of ventricular contraction was said to occur. Inspection of their records reveals only a small and probably insignificant increase in amplitude, occurring with an acceleration in rate.

The alleged digitalis-like action was observed on the frog's heart (37-38) the authors concluding that the difference between digitalis and barium chloride was merely quantitative—the criterion was systolic and diastolic standstill of the frog's heart. The barium chloride concentrations used were extremely high ranging from 1:10 to 1:12,000. Systolic standstill occurred with high concentra-

tions (1 200 and higher) and diastolic arrest occurred with the lower concentrations. The practical and theoretical significance of the results obtained with such high concentrations is questionable. In the present study most of the experiments were performed around the maximum concentration range attainable in the blood after large therapeutic doses in man, viz, 1 50,000. A preliminary report was published elsewhere (31).

*Results* In concentrations between 1 100,000 and 1 10,000 barium chloride caused no significant changes in the contractile force of the papillary muscle preparation. In higher concentrations (1 5,000 to 1 1,000) diminution of force occurred almost immediately, varying in degree with the concentration. The toxic effect could be partially reversed with Locke's solution. In no instance was spontaneous activity induced (total of 12 experiments), i.e., the muscle contractions corresponded with the frequency of electrical stimulation.

*Comment* It appears from the results reported here that barium chloride has no "digitalis-like" action on the force of contraction of mammalian cardiac muscle, and that in all probability small increases in the amplitude of ventricular contraction observed by Poulsson (37) and van Egmond (36) are secondary to cardiac acceleration. The systolic and diastolic arrest noted by Poulsson (37) and Werschlin (38) are probably due to a non-specific toxic effect since the concentrations causing them are quite high and it does not necessarily signify a "digitalis-like action." In the present experiments, the absence of spontaneous rhythmicity due to barium even in high concentrations is not explained, however, it is possible that factors involving the intact circulation are prerequisite.

III THE XANTHINES CAFFEINE, THEOBROMINE AND THEOPHYLLINE Experiments using human subjects (39 and others) have shown substantial increases in cardiac output after therapeutic doses of the xanthines, but simultaneously there occurred a considerable decrease in peripheral resistance and increase in arteriovenous oxygen difference. Grollman (40) has observed an increase in oxygen consumption of about 35 per cent following large doses of caffeine orally in man, and has stated that whether the cardiac output is increased or not depends on whether the arteriovenous oxygen difference varies inversely.

Numerous investigators have studied the action of theobromine, theophylline, and especially caffeine, on isolated heart preparations. It has long been known that the amplitude of contraction of the isolated frog heart (41, 42) and of isolated mammalian heart preparations (42, 43, 44) is increased by suitable concentrations of a xanthine in the perfusate, this effect has been considered evidence of a direct myocardial stimulant action. Hedbom (43), Bock (44), Loeb (45) and others, using the Langendorff mammalian heart preparation, obtained data which were in substantial accord regarding the action on rate, amplitude and coronary flow, viz, increase in rate and amplitude, and also increase in coronary flow, sometimes with concentrations at which rate and amplitude were unaltered. Plant (46) likewise noted that output and amplitude in the Knowlton Starling heart-lung preparation was increased, but stated that the output per beat was unchanged. Almost invariably increased amplitude caused by the xanthines in dilutions of 1 10,000 or thereabouts was accompanied by increased

rate. Inasmuch as the amplitude or force of contraction of cardiac muscle increases with the rate (47) it is difficult to decide which is the primary action. Undoubtedly both direct myocardial stimulation and that consequent to changes in rate operate but the proportions contributed by each are not apparent. Heathcote (42) found that the coronary flow in the isolated mammalian heart preparation was increased along with rate and amplitude by concentrations of the xanthines of about 1:5,000. This is in substantial accord with the work of others. This is another factor operating to alter the force of the heart and it

TABLE 1

*Effect of the xanthines on tension developed by cardiac muscle*

DRUG	CONCENTRATION	NUMBER OF EXPERIMENTS	AVERAGE TIME OF ONSET	AVERAGE MAXIMUM INCREASE† (PER CENT OF CONTROL)	AVERAGE TIME TO MAXIMUM TENSION INCREASE†	AVERAGE DURATION OF ACTION†
			minutes		seconds	minutes
Caffeine	1:5,000	6‡	2	14	5	3
	1:2,000	5‡	1	21	3	3
	1:1,000	9‡	1	34	1½	12
	1:500	6	Immediate	50	4	17
	1:250	6	Immediate	104	5	10
Theobromine	1:10,000	4	Immediate	24	2	3
	1:5,000	5¶	Immediate	78	2½	12
	1:2,000	4	Immediate	116	2	8
	1:1,000	4	Immediate	148	4	8
Theophylline	1:20,000	7‡	1½	35	2½	11
	1:10,000	6	3½	75	10	20
	1:5,000	8	Immediate	88	3	20
	1:2,000	4	Immediate	154	2	24

Average of positive experiments only

† Average of all experiments

‡ 4 experiments were negative.

§ 3 experiments were negative

¶ 1 experiment was negative

|| 2 experiments were negative

necessarily makes uncertain the implication of a direct cardiac muscle action. Heathcote stated that his results made it difficult to hold that rate and amplitude increases caused the increased coronary flow although the reverse situation could hold. A. J. Clark (48) states that the pace-maker (mammalian heart) is stimulated by caffeine.

In the present study the drugs were used in the form of free base whenever possible, but when high concentrations were required, caffeine sodium benzoate, theobromine sodium salicylate and theophylline ethylenediamine or sodium acetate were used. A preliminary report has appeared elsewhere (49).

**Results** The results are summarized in table 1. Graphs of representative

experiments appear in figure 1. Stimulation by concentrations lower than those indicated in the table was not observed. Considerable variation in magnitudes of response occurred at a given concentration of any of the drugs, this being attributable, at least in part, to varying degrees of failure in different preparations. When the xanthine solution was replaced by Locke's solution during the height of its effect, in the cases in which the action was relatively prolonged, there was very rapid resumption of the original force by the muscle.

(a) *Caffeine* In concentrations between 1/2,000 and 1/5,000 caffeine produced an increase in "systolic" tension in less than half the experiments and lower concentrations were without effect. Increases in tension of 50 to 250 per

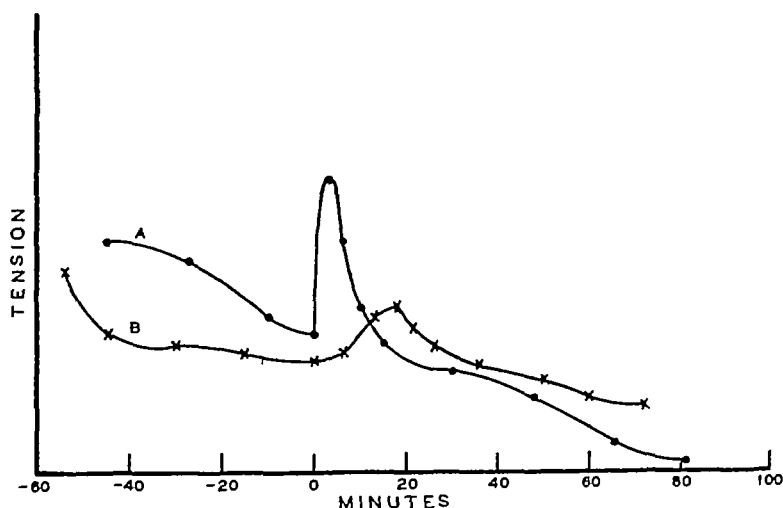


FIG. 1. GRAPHS OF EXPERIMENTS ILLUSTRATING THE ACTION OF CAFFEINE ON THE FORCE OF CONTRACTION OF ISOLATED MAMMALIAN CARDIAC MUSCLE.  
Drug applied at 0 time. A, 1/1,000; B, 1/2,500.

cent occurred after concentrations of from 1/1,000 to 1/250, starting in from 2 to 15 minutes after the drug was applied, with a maximum shortly thereafter. The duration of action rarely exceeded 30 minutes, the higher concentrations causing inexcitability and rigor in 15 or 20 minutes.

(b) *Theobromine* In concentrations of less than 1/5,000, no significant changes in "systolic" force were observed, and in 1/5,000 concentration augmentation of the tension was rare, the majority of experiments showing equivocal changes. Higher concentrations had a more marked effect. As with caffeine, onset of the action was rapid and duration was brief.

(c) *Theophylline* This drug is the most effective of the xanthine group in increasing "systolic" force. In concentrations of 1/10,000 to 1/5,000, the tension may be increased from 50 to 250 per cent and in some experiments 1/20,000 was effective. The onset of action is as rapid as with caffeine and theobromine,

and the duration of action is generally longer. In one case, the 1:5,000 caused a 100 per cent increase 90 minutes in duration, in this case it was demonstrated that replacement of the drug with fresh Locke's solution resulted in an immediate reduction of the 'systolic' tension to the control level. The sequence and time relations of events following higher concentrations were similar to those after caffeine and theobromine.

There was no apparent correlation between the action of any of the xanthines and changes in 'tone' or resting length of the muscle, and emphasis should be placed on the fact that replacement of the drugs by fresh Locke's solution abolished the action immediately. Occasionally high concentrations caused spontaneous activity i.e. a rate of contraction higher than that resulting from the regular stimulation. No difference between the effects of free bases and the salts was observed.

*Comment.* It is evident that the xanthines do not cause a significant increase in the 'systolic' tension of heart muscle in concentrations attainable in the blood of man after therapeutic doses even when given by intravenous injection. If one considers the fact that the xanthines disappear from the blood stream very rapidly (Hatcher and Kwit, 50) considerable caution must be exercised in accepting such a measure as cardiac output as a valid criterion of direct myocardial stimulation and persistence of cardiac action. This disappearance from the blood is presumably due to metabolism and diffusion into and dilution in the interstitial fluid and is important in the case of xanthines for replacement of the Locke's-xanthine solution by Locke's in these experiments terminates their action i.e., their action does not persist. In the present experiments the short duration of action during continued exposure to effective concentrations of the xanthines may possibly be explained by accelerated failure of the muscle resulting from increased resting metabolism (51).

From these experiments it appears that the dose necessary to produce significant, but temporary myocardial stimulation in man would need to exceed that causing undesirable side effects on the central nervous system metabolism and peripheral circulation.

**IVA. EPINEPHRINE.** Among the circulatory actions which epinephrine is known to exert, that on the heart has been subjected to considerable experimental scrutiny. As in the cold blooded animal its action in accelerating and increasing the amplitude of the mammalian heart has long been known. For example, the amplitude of ventricular excursions is increased accompanying the rise in blood pressure in dogs after intravenous injection of 0.02 mg per kg (38). The effects of noxious agents (chloroform, chloral hydrate) on the excised cat's heart were 'reversed' by epinephrine, also excised cat hearts which had been refrigerated for 24 hours could be revived by epinephrine (52). That the acceleration by epinephrine is not dependent on connections with the central nervous system was shown in the denervated cat's heart, in addition the use of quiescent ventricular strips has shown that the drug can cause spontaneous rhythmicity to appear thereby suggesting that acceleration can occur by an action directly on cardiac muscle, exclusive of an action on the pace-maker (53).

The presence of sympathetic innervation may be necessary for susceptibility to epinephrine<sup>4</sup>

In an analysis of acceleration and "strengthening" of the heart beat by epinephrine (54), it was found that doses causing marked vasoconstriction increased the amplitude with concomitant cardiac slowing, even when the blood pressure was maintained at a constant level. This demonstrated that the force of the heart may be increased by epinephrine in the absence of elevated aortic pressure. A more direct approach to this question was made by Wiggers (55), who maintained the peripheral arterial resistance at a constant value by means of an adjustable aortic clamp, "drove" the heart at a constant rate, and recorded

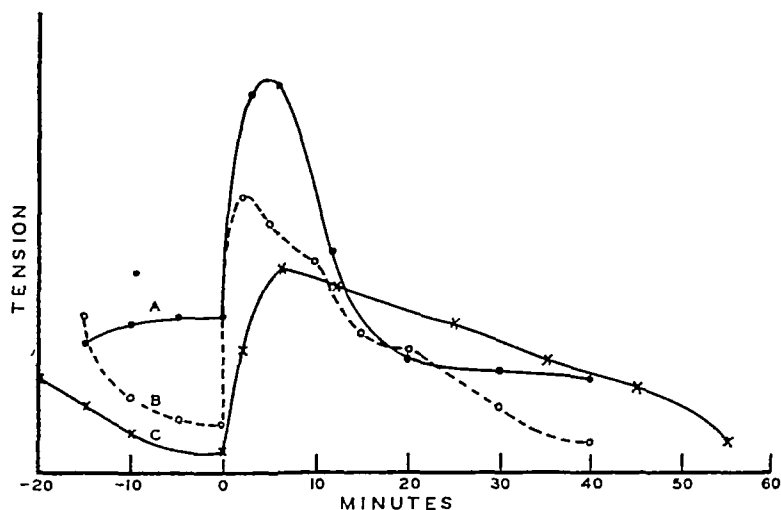


FIG 2 GRAPHS OF EXPERIMENTS ILLUSTRATING THE ACTION OF EPINEPHRINE ON THE FORCE OF CONTRACTION OF ISOLATED MAMMALIAN CARDIAC MUSCLE

Drug applied at 0 time A, 1 100 million, B, 1 10 million, C, 1 1 million

left intraventricular pressure changes by means of an optical manometer. Epinephrine steepened the presphygmic isometric pressure gradient, likewise steepening the relaxation (postsphygmic) gradient. The course of events during the ejection period was not proportionately altered, and the duration of total systole was shortened. He concluded that epinephrine stimulates the ventricle by increasing contraction and relaxation gradients, by augmenting the maximum systolic pressure, and by shortening systole. Since the intraventricular pressure was "far below the normal level at the very beginning of diastolic inflow," he also concluded that epinephrine acted specifically on the relaxation process. However, the extent to which this latter phenomenon depends on more complete and more rapid ejection of ventricular contents was not ascertained.

<sup>4</sup> Recently, Hiatt et al (*Am J Physiol*, 133: 753, 139-45, 1943) found the amplitude (and rate) of the cold blooded heart in the absence of sympathetic innervation were increased, but believe the effects are greater in its presence.

**Results** On the isolated mammalian papillary muscle epinephrine exerts a characteristic augmentation of the contractile force. Graphs of representative experiments of the present study are presented in figure 2 and a summary in table 2. Epinephrine in concentrations as low as 1:200 million seldom fails to elicit an increase in tension varying from 50 to 400 per cent, together with a marked shortening of the duration of contraction. The effect is almost im-

TABLE 2  
*Effect of epinephrine on tension developed by cardiac muscle*

CONCENTRATION	NUMBER OF EXPERIMENTS	AVERAGE MAXIMUM TENSION INCREASE (PER CENT OF CONTROL)	AVERAGE TIME FOR MAXIMUM TENSION INCREASE	AVERAGE DURATION OF ACTION
			minutes	minutes
1:200 million	7	216	2	14
1:100 million	6	297	1½	19
1:10 million	7	588	1½	21
1:1 million	5	1258	1½	24
1:100 000	4	Depression		
1:50 000	3	Depression		
1:10 000*	4	Depression		

Duration of action of or recovery from these concentrations was not studied.

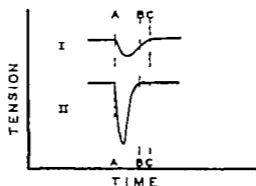


FIG. 3 PHOTOGRAPHIC TRACING SHOWING THE INFLUENCE OF EPINEPHRINE ON 'SYSTOLIC' TIME OF THE CAT'S PAPILLARY MUSCLE DRIVEN AT A CONSTANT RATE

Downward excursion represents contraction

I Before epinephrine II After epinephrine 1:10 million.

Note that 'systole' is shortened by approximately one-third (A-B compared with A-C) that the slopes of contraction and relaxation are increased and that the 'systolic' force is trebled

mediate attains its maximum usually in about 2 minutes and lasts for 10 to 20 minutes. The effect may be repeated several times in a given preparation, and its magnitude depends within wide limits, on the concentration. A given concentration brings about the same response on repeated application. Higher concentrations (1:10 million to 1:1 million) cause an increase in systolic force which may be as high as 15 to 18 times the initial control level. The duration of the effect is usually 20 to 30 minutes under such conditions, although occasionally it may last only 5 minutes. Frequently such high concentrations

cause spontaneous rhythmicity of the preparations, which is usually, though not always, regular in rate. Toxicity, evident as a reduction in "systolic" force, occurs with concentrations 1 100,000 and higher, and is usually reversible. In any of the concentrations used, the action could be promptly terminated by replacing the drug solution with unmodified Locke's solution.

Even in concentrations causing minimal increases in "systolic" tension, epinephrine causes shortening of "systolic" time. In two experiments in which high concentrations (1 10 million and 1 1 million) were used, the systolic interval was measured and compared with the control values, the "systolic" period was reduced by one-third, with a 3- and 10-fold increase in force. In two experiments lower concentrations (1 100 million) caused a similar reduction in "systolic" time, with a 2- to 8-fold increase in "systolic" tension. Figure 3 illustrates the effect. In one experiment in which the preparation responded to alternate stimuli only, epinephrine in 1 20 million restored the excitability, and as the effect wore off the original loss of response to alternate stimuli was reestablished. The increase in "systolic" force occurred before the excitability recovered. An increase in resting length ("diastolic") is especially noticeable with higher concentrations (1 1 million or 1 10 million). With lower concentrations this is manifested as an interruption in the progressive shortening coincident with spontaneous failure during the control period.

*Comment.* It is evident from the foregoing data that epinephrine increases the force of the heart in consequence of a direct action on cardiac muscle which is independent of alterations in cardiac function secondary to the known peripheral circulatory changes which epinephrine induces. This is in agreement with the interpretations of previous workers. The depressant effects of very low concentrations observed by Sollmann and Barlow (56) on the frog heart were not seen in the present study on mammalian heart muscle. The increased intraventricular pressure and shortening in the systolic time found by Wiggers finds confirmation in the increased "systolic" tension and shortening of the "systolic" interval in the present experiments.

The direct action of epinephrine on the heart conforms with the emergency theory of the function of this hormone in that it provides for increased activity under conditions of stress.

**IVB. EPHEDRINE.** The cardiovascular actions of ephedrine were first studied by Miura (57) on the frog heart and on mammals by Chen and Schmidt (58). The latter demonstrated responses which classified ephedrine as a "sympathomimetic" drug, in the sense that the drug produced effects similar, in many respects, to those obtained after excitation of organs and musculature innervated by sympathetic nerves. The results of studies prior to those of Chen and Schmidt indicated that its primary action on the frog heart was depression of rate and amplitude in high concentrations (1 10<sup>6</sup>). Lower concentrations (1 10<sup>7</sup>) were largely without effect, although in a few instances a slight increase in frequency and amplitude was observed (59). On the other hand, others (60, 61) have observed stimulation by ephedrine in "dilute solutions", Kreitmar (61) likewise noted that atropine did not prevent the depression by stronger solutions

of ephedrine, establishing that the action was not via the nervous cardioinhibitory mechanism

Chen and co-workers (58-62) noted that the isolated perfused rabbit heart exhibited the same behavior as the frog heart toward ephedrine in relation to concentration rate and amplitude were increased by concentrations of 1:100,000 whereas a decrease in these functions occurred during perfusion with 1:10,000 to 1:5,000. Higher concentrations produced varying degrees of block and eventually failure. The results were amply confirmed by others (63, 64). Small doses in cats and rabbits increase ventricular contraction whereas large (5 mg.) doses depress the ventricle (61-65). Chen and Schmidt (58) found a marked increase in right ventricular amplitude after ephedrine. Coincident with cardiac acceleration and rise in blood pressure an increase in cardiac output was observed in anesthetized and atropinized dogs (62, 66). Chen and Schmidt (67) concluded that the cardiac stimulant effects from small doses are 'fully comparable with those of excitation of the accelerator nervous mechanism of the heart'. The effects are much less pronounced than those of epinephrine, and, due to the depressant action of large doses the effects cannot be increased with dosage as in the case of epinephrine. They therefore consider it inadvisable to inject ephedrine intracardially in an emergency.

**Results** Ephedrine in concentrations of 1:500,000 to 1:200,000 caused an increase of from 25 to 100 per cent in 'systolic' force of the isolated papillary muscle lasting from 10 to 20 minutes. This was followed by depression, an effect which was partially reversible by Locke's solution. Lower concentrations were without demonstrable effect, whereas higher concentrations were purely depressant. In concentrations of 1:20,000 to 1:10,000 the substance was very toxic and the response failed rapidly. If the depression did not proceed too far it could be partially reversed by unmodified Locke's solution. When a preparation had once been 'stimulated' by a low concentration of ephedrine subsequent additions of ephedrine of equal or higher concentrations failed to elicit the response (observed several times in 4 experiments; a fifth experiment gave uncertain results due to rapid muscular failure). Reversibility of this effect could not be conclusively demonstrated by washing out with Locke's solution, the results being almost evenly divided (in 2 out of 5 experiments, a partial return of response was observed).

The effect of ephedrine on epinephrine action was studied in 5 experiments. Control effects of 1:200 million and 1:100 million epinephrine were obtained and again observed after exposure of the muscle preparations to various concentrations of ephedrine, ranging from 1:500,000 to 1:10,000. Ephedrine in 1:500,000 concentration had no effect on the responses to epinephrine in the above concentrations but 1:100,000 prevented the response to 1:200 million epinephrine in 3 experiments, and ephedrine in 1:10,000 prevented the response of 1:1 million epinephrine in 3 experiments.

**Comment** The results obtained indicate that ephedrine exhibits the phenomenon of 'tachyphylaxis' in its action on heart muscle as has been reported for its action on blood vessels. This may be interpreted as additional evidence in con-

firmation of the theory of Gaddum and Kwiatkowski (68) and Morton and Tainter (69), that ephedrine is dependent for its action on the presence of epinephrine

Since the total quantity of enzyme or enzymes which normally inactivate epinephrine is small in proportion to the large excess of epinephrine in the immersion medium under the conditions of the present experiments, it is not surprising that "sensitization" of the muscle preparation to epinephrine was not demonstrable and need not signify that such sensitization does not in fact occur in the heart of the intact animal

In higher concentrations ephedrine is definitely depressant by a primary action on heart muscle, and this observation emphasizes Chen and Schmidt's warning against its use by intracardiac injection. Whether the prevention of epinephrine action by high concentrations of ephedrine is related to the depression by ephedrine alone cannot be determined from the present experiments, but the phenomenon may find explanation in the hypothesis of Curtis (70) and of Gaddum and Kwiatkowski (68) who suggest that high concentrations of ephedrine occupy the epinephrine receptors in the cell, thereby preventing the action of epinephrine

#### SUMMARY

The property of producing sustained augmentation of the "systolic" force of mammalian cardiac muscle which is characteristic of the digitalis glycosides is absent in camphor, pentamethylene tetrazol ("Metrazol," "Cardiazol"), nikethamide (pyridine- $\beta$ -carboxylic acid diethylamide, "Coramine"), and barium chloride

The xanthines (caffeine, theobromine and theophylline) are ineffectual except in concentrations considerably above those attainable therapeutically. Compared with digitalis glycosides, their action is rapid in onset and brief in duration

Epinephrine augments the "systolic" force of mammalian heart muscle to a marked degree and in concentrations attainable with therapeutic doses. The effect is almost immediate in onset, its duration is a matter of only about 10 to 20 minutes rather than hours as in the case of the digitalis glycosides

Therapeutically attainable concentrations of ephedrine may increase the force of cardiac muscle. The range between the concentration which stimulates and that which depresses the heart muscle is small. Under the conditions of the present experiments it did not "potentiate" epinephrine action, but rather reduced it

*Acknowledgment* The author wishes to express his gratitude to Dr. McKen Cattell for guidance and for suggesting the problem, and to Dr. Harry Gold for many valuable criticisms

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# THE INTERACTION BETWEEN PROCAINE, COCAINE ADRENALINE AND PROSTIGMINE ON SKELETAL MUSCLE

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Evidence that procaine has a curare-like action upon skeletal muscle has been obtained by several workers Liljestrand and Magnus (1), Fulton (2) and Brumer and Titeca (3) Ruesser and Neuschloss (4) found that procaine abolished the response of frog muscle to the application of acetylcholine and Frank, Nothmann and Hirsch Kauffmann (5) showed that procaine diminished the response of denervated skeletal muscle to acetylcholine and nicotine Recently Harvey (6) has pointed out several respects in which the action of procaine resembles that of curare. When injected into the artery just above the point where the artery enters the muscle small doses of procaine reduce the response of the muscle to maximal single shocks applied to the motor nerve and also the tetanic response to a series of shocks but they do not reduce the response to direct stimulation. When the response to single shocks has been depressed by procaine, brief tetanic stimulation of the nerve augments the response, as it does when depression is produced by curarine. Procaine also depresses the contraction resulting from the injection of acetylcholine as does curarine, but again like curarine, does not affect that produced by the injection of KCl Harvey observed in addition that when procaine is injected into a muscle during eserine potentiation the twitches are immediately reduced when eserine is administered to a muscle previously injected with procaine, the potentiating effect of eserine is replaced by a slight depression Harvey carried out experiments on the perfused superior cervical ganglion of the cat he observed that procaine like curarine, prevented the transmission of impulses through the ganglion but had a second action not possessed by curarine of arresting the output of acetylcholine during preganglionic stimulation. Procaine also abolished the stimulating action of acetylcholine on the ganglion and depressed that of KCl

Although procaine has this curarine-like action, there are no clinical reports indicating that such an action has ever been observed in the course of using procaine as a local anaesthetic in subjects with a normal neuromuscular mechanism In patients suffering from myasthenia gravis however Carmichael (7) found that procaine, used for infiltration anaesthesia, caused a great increase in muscular weakness. Since myasthenic patients are treated with prostigmine, we have investigated the action of procaine in relation to prostigmine for recent evidence obtained by Bülbring and Burn (8) shows that prostigmine and eserine are not identical in action and it seemed possible that Harvey's observations on procaine and eserine might not apply to procaine and prostigmine Observations

have also been made on the action of cocaine which has been found to possess the same action as procaine and to exert it in smaller dose

**METHOD** Cats were used, either spinal, or anaesthetised with chloralose. The contractions of the left gastrocnemius muscle were recorded isometrically. Single maximal shocks (condenser discharges from a neon lamp circuit) were applied to the sciatic nerve at various rates. Unless otherwise stated, all injections were given intra arterially into a cannula inserted into the right iliac artery pointing towards the aorta, so that the drug was carried with the bloodstream down the left leg. The animals were prepared in the same way as that described by Bülbring and Burn (8). All solutions were made in 0.9% saline.

Prostigmine was used as methyl sulphate (kindly supplied by Roche Products Ltd), cocaine and procaine both as hydrochlorides, curarine as chloride.

**EXPERIMENTAL RESULTS** Perhaps the clearest difference between the action of procaine and that of curarine is that procaine has little effect on the contractions of normal muscle elicited through the nerve, whereas curarine has a great effect. On the other hand procaine was found to have its main depressant action only after a previous injection of prostigmine. The record in figure 1 shows the contractions of the gastrocnemius muscle of a cat in response to 15 stimuli per min. The large and rapid increase in the size of the contractions caused by the injection of 30  $\mu$ g prostigmine is well shown, and then the sudden decrease taking place when 25 mg procaine was injected. It was evident that a far greater diminution occurred in contractions augmented by prostigmine than in normal contractions, for as shown in figure 2 (C), taken from the same experiment, the injection of 25 mg procaine had by itself a scarcely perceptible effect. The record in figure 2 (C) shows further that procaine not only abolishes, but also, if given before, prevents the action of prostigmine in causing augmentation. The difference is brought out again in figure 3 taken from an experiment in which curarine was infused intravenously at such rate as to maintain the muscle response to nerve stimulation at about one third of its normal height. The contraction before curarine was injected is shown in figure 3, A, the contraction during the infusion of curarine is shown in figure 3, B, and in this part of the record it is seen that the injection of prostigmine was not without effect, but at once augmented the contractions in the usual way. In figure 3, C, however, procaine was injected first, yet although the muscle contractions were scarcely reduced, the action of prostigmine was abolished.

A further difference between procaine and curarine was found in their effect in the presence of prostigmine, for after the injection of prostigmine the action of curarine was much reduced. In figure 4 (A) is shown the reduction in the muscle contractions produced by the injection of 0.7 mg curarine. This effect was allowed to pass off, and 30 minutes later, 0.06 mg prostigmine was injected. A repetition of the injection of 0.7 mg curarine after one minute now produced a much smaller effect. This diminution of the action of curarine by prostigmine was repeatedly observed, and it was seen to persist after the effect of prostigmine on the muscle tension had disappeared.

**The action of adrenaline** Bülbring and Burn (8) have shown that in the presence of prostigmine, adrenaline often causes a further augmentation of muscle contractions. A good example of this is seen in figure 1 (B). When

20  $\mu$ g adrenaline was injected shortly after 30  $\mu$ g prostigmine the effect of the prostigmine was almost doubled. The tracing in figure 1 (A) shows that when procaine had abolished all but a small part of the effect of prostigmine some augmentation of the contractions was still produced by adrenaline. Likewise

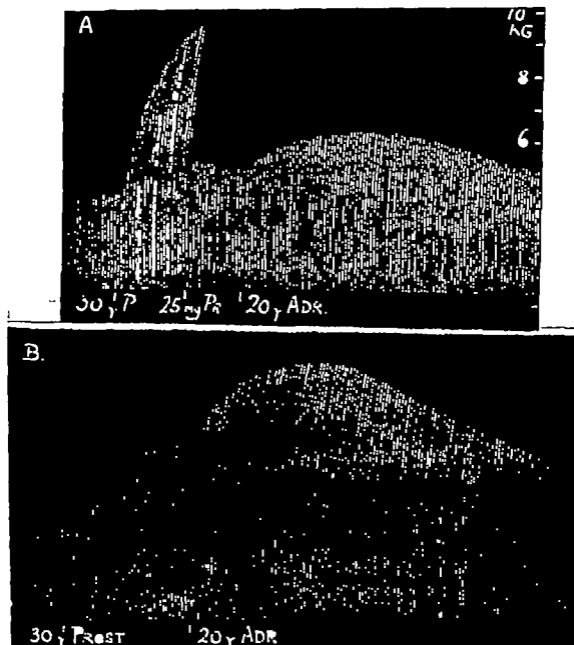


FIG 1 Cat anaesthetised with chloralose. Contractions of gastrocnemius muscle stimulated by single maximal shocks applied to the sciatic nerve 16 per minute. A shows the augmentation of muscle contractions by the intra-arterial injection of 30  $\mu$ g prostigmine and the removal of most of the augmentation by 25 mg procaine. The injection of 20  $\mu$ g adrenaline still causes some augmentation. B shows the effect of injecting prostigmine and then adrenaline without the interposition of procaine.

as seen in figure 2 (C) when the previous injection of procaine had prevented the action of prostigmine adrenaline still produced an augmentation.

There is a further difference in the action of procaine according to whether prostigmine is present alone or together with adrenaline. In figure 2 (A)

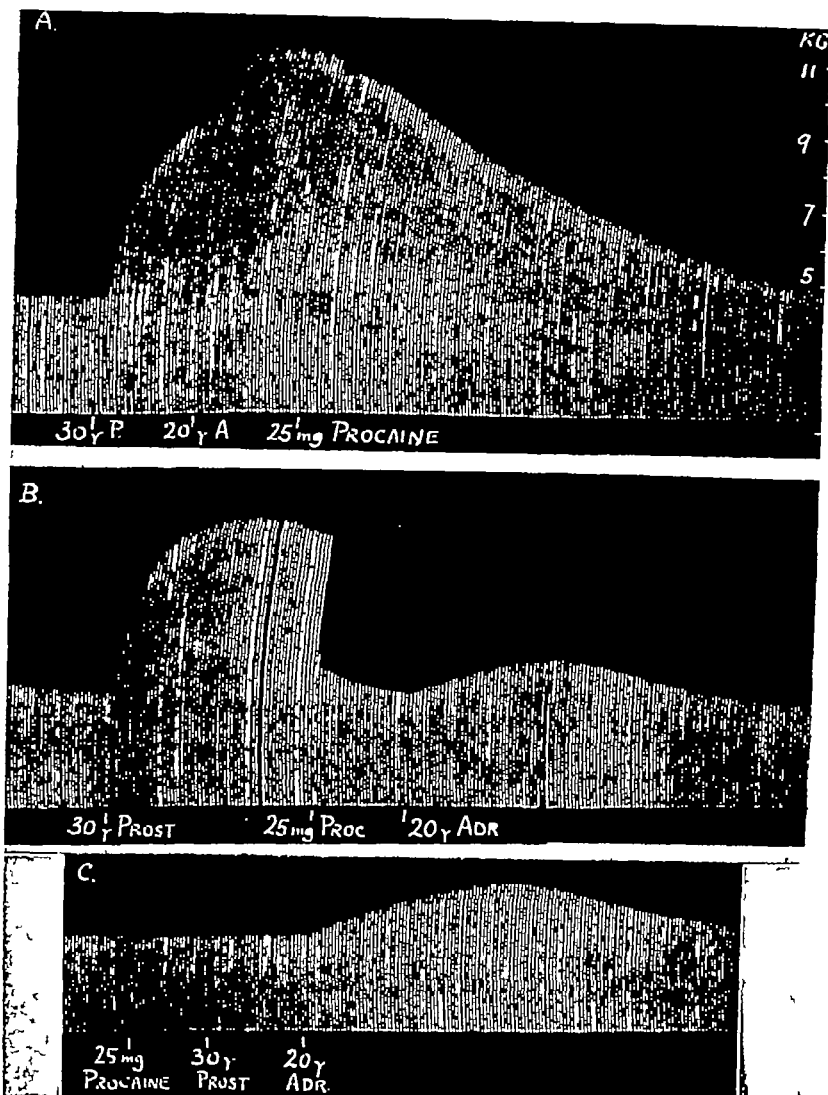


FIG. 2 Same experiment as figure 1 continued. In A the injection of  $20\mu g$  adrenaline after the prostigmine inhibits the action of procaine seen in figure 1 A. That this inhibition is not due to the longer time interval between the injection of prostigmine and procaine is seen in B. In C note that 25 mg procaine does not depress the normal muscle contractions, but does prevent prostigmine from having its usual effect. It does not abolish the adrenaline effect however.

adrenaline was interposed between prostigmine and procaine with the result that the inhibitory action seen in figure 1 (A) was scarcely evident. That the effect

of procaine was so slight was not due to the difference in time interval, as is seen in figure 2 (B) where the effect of procaine is similar to that in figure 1 (A)

With a rate of stimulation of 15 per minute or more adrenaline given after prostigmine instead of producing an augmentation frequently causes a depression

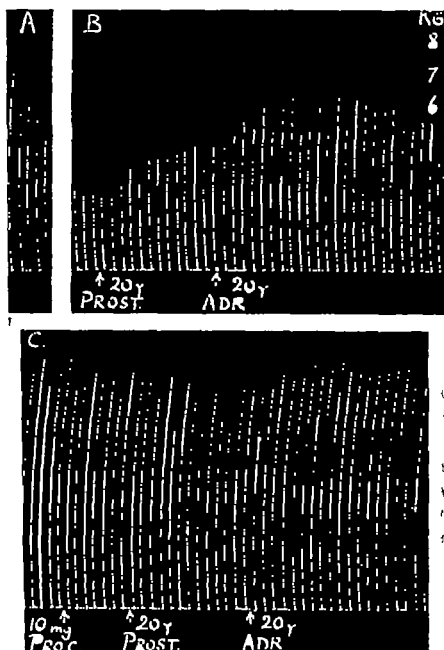


FIG 2 Spinal cat. Records as figure 1. Stimulation 5 per minute. Comparison between the action of curarine and procaine. A shows the size of contractions before, B the effect of 20 μg prostigmine followed by 20 μg adrenaline during curarine infusion. C after 10 mg procaine.

of muscular contractions attributed by Bülbring and Burn (8) to acetylcholine paralysis. The amount of acetylcholine greater because of the faster rate of stimulation and because of the presence of prostigmine, has its action intensified by adrenaline so that the effect of excess results. This is illustrated in figure 5 (A). After the injection of cocaine however when prostigmine no longer

augmented the contractions (figure 5 (B)) the action of adrenaline was reversed, so that instead of the depression an augmentation was observed. Precisely similar records were obtained when procaine was injected.

*Procaine on prostigmine depression* It has been shown by Briscoe (9) and Bacq and Brown (10), that when the muscle is treated with eserine and stimulated through the nerve at a fast rate, the tension declines rapidly. In the experiments to be described prostigmine was used instead of eserine, and the same

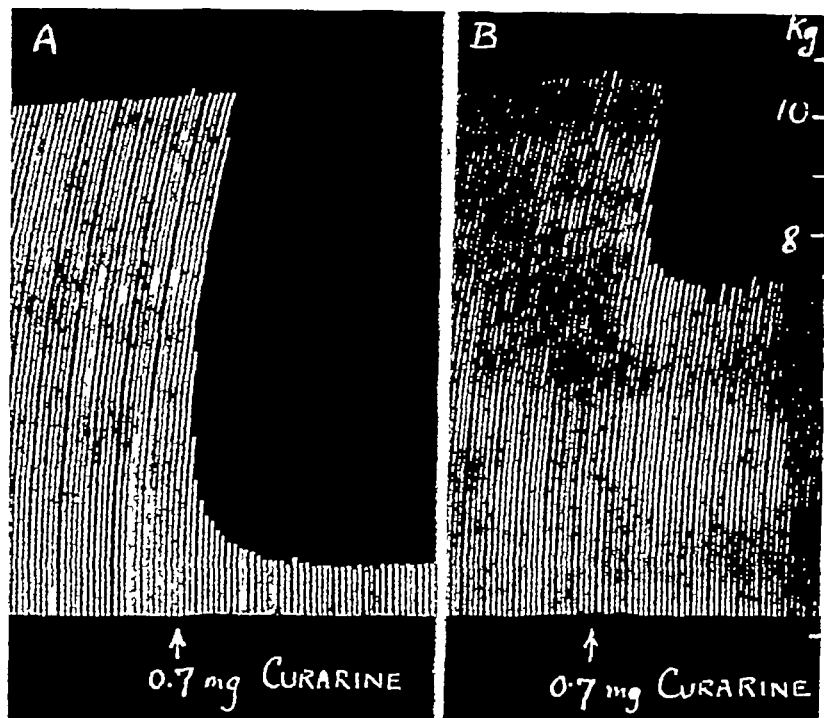


FIG 4 Spinal cat. A shows effect of injecting 0.7 mg curarine. B shows the much smaller effect of the same dose of curarine injected 2 minutes after 0.06 mg prostigmine.

results were observed. The nerve was stimulated at the rate of 4-5 per minute. Every five minutes the rate of stimulation was altered to a faster rate for a period of one minute. A rate of stimulation was chosen which did not itself produce a fall in tension through fatigue, and this was repeated after a dose of prostigmine, when the contractions during fast stimulation rapidly declined. A dose of procaine injected after the prostigmine prevented the decline and during the period of fast stimulation the muscle contractions remained similar to those before. Figure 6 illustrates this. (A) shows a control observation of a period

of 90 stimuli per minute (B) in the presence of prostigmine, shows a rapid decline of tension to below the original height as soon as the fast stimulation starts, and that the tension becomes at once greater with the slower rate. In (C) after 10 mg procaine the muscle is again capable of maintaining the height of

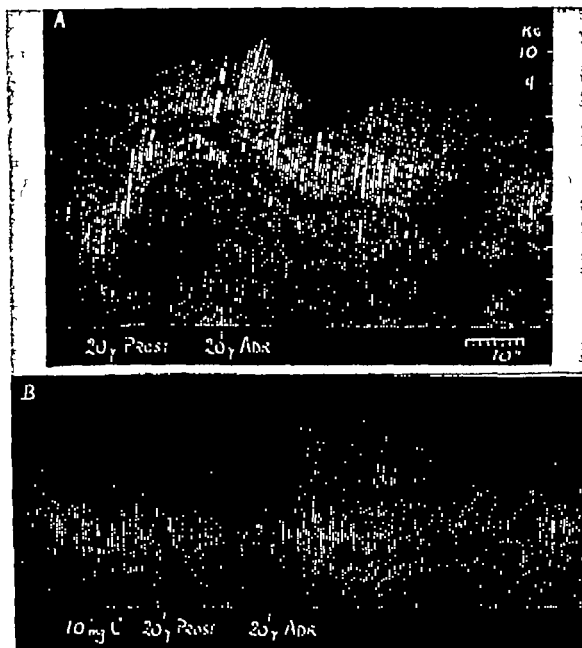


FIG 5 Cat chloralose. Records as figure 1. Rate of stimulation 14 minute. A shows the depression of muscle tension caused by adrenaline given after prostigmine. In B 10 mg cocaine prevented the action of prostigmine and converted the adrenaline depression into a potentiation.

contractions during the period of fast stimulation. When comparable observations were made with curarine, it was found to have the same action as procaine and cocaine. Muscle contractions are however depressed by larger doses of prostigmine even when the nerve is stimulated at a slower rate. Experiments

were carried out in which the muscle contractions were depressed by single injections of prostigmine and also by constant intravenous infusion of prostigmine. In none of these experiments did procaine abolish or even lessen this depression.

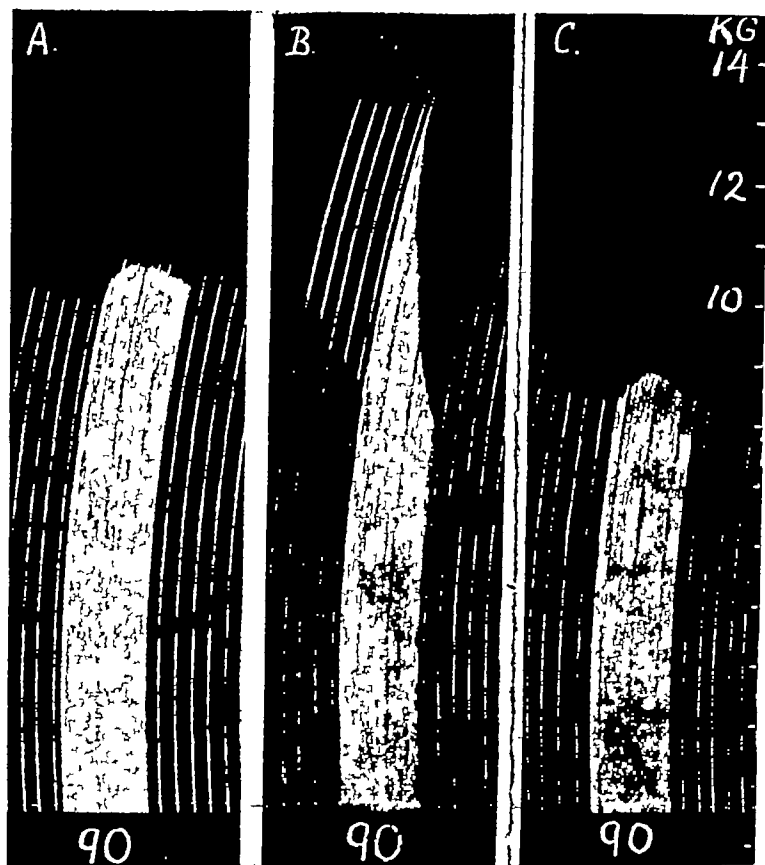


FIG 6 Spinal cat. Records as figure 1. One-minute periods of fast stimulation (90 per minute). A before B after  $40\mu\text{g}$  prostigmine C after 10 mg procaine. Note the rapid fall in tension in B which is prevented in C.

**Acetylcholine depression** Bacq and Brown (10) found that a dose of acetylcholine interpolated in a series of maximal stimuli caused a depression of muscle contractions in the presence of any anti-cholinesterase. This effect can be seen in figure 7 (A). When acetylcholine was injected intra-arterially into a muscle contracting in response to maximal nerve stimuli and previously treated with prostigmine, the size of contractions was decreased. After the injection of 2.5 mg cocaine the depression was scarcely evident as seen in figure 7 (B). As the

effect of acetylcholine depended partly on the amount of prostigmine present, the experiments were repeated with an intravenous infusion of prostigmine. The rate of infusion was adjusted so that the size of the contractions remained constant and the effect of an intra-arterial dose of acetylcholine was then also constant. A similar failure of acetylcholine to act in the presence of cocaine or procaine was seen in these experiments. Harvey (6) observed that in normal muscle the twitch following on an intra-arterial injection of acetylcholine was reduced by procaine. In our experiments the more prolonged acetylcholine

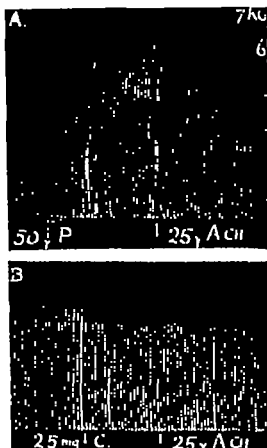


FIG 7 Cat chloralose. Records as figure 1. Rate of stimulation 12 per minute. The depression of muscle tension produced by acetylcholine in the presence of prostigmine A is abolished by 2.5 mg cocaine B.

contraction in the presence of prostigmine was also reduced by cocaine or procaine as may be noticed in figure 5.

**DISCUSSION** Harvey (6) has published evidence supporting the view that procaine has an action on neuromuscular transmission like that of curarine and we are in full agreement. As might be expected there are some points of difference in the action of procaine and curarine and our observations concern themselves in the main with these differences. When muscle is stimulated through the nerve curarine has a much greater inhibiting effect on the normal contractions than it has on the contractions after the injection of prostigmine. On the other hand procaine has very little effect on normal contractions, but greatly reduces the contractions after the injection of prostigmine. Undoubtedly

this observation explains the collapse which has been observed when procaine has been used for infiltration anaesthesia in patients suffering from myasthenia gravis who are constantly taking prostigmine. The same difference between procaine and curarine is seen in another way, after the injection of procaine, although the muscle contractions are undiminished, prostigmine is without effect, after the injection of curarine, although the contractions are reduced, prostigmine produces an augmentation.

Bülbring and Burn (8) have shown that when muscle contractions are increased by prostigmine the injection of adrenaline causes a further increase. We have found that when procaine is injected so that prostigmine produces no augmentation of muscle contractions, adrenaline following the prostigmine still augments the contractions. Furthermore if muscular contractions are augmented by prostigmine, the injection of adrenaline then greatly reduces the effect of procaine if this is given after the adrenaline. This relation between adrenaline and procaine is not surprising if the action of procaine is considered to resemble that of curarine. The anti-curarine action of adrenaline is well known.

Since procaine has so little action on normal muscle contractions, and so much on those augmented by prostigmine, an alternative to describing procaine as a curarine-like substance would be to describe it as a substance having a specific neutralising action for prostigmine. The evidence indicates that this description would be wrong, for procaine does not abolish or diminish the effect of prostigmine when this is given in amounts which depress muscle contractions.

Various observations have been made that procaine and cocaine have a sympathomimetic action, and also that they either increase or diminish the action of adrenaline. Recent observations of this kind have been made by Macgregor (11), Tripod (12), Tripod and Chakravarti (13) and Phulpot (14). In our work no results were obtained which indicated that procaine or cocaine might be exerting a sympathomimetic action, their effect was indeed reduced or abolished by adrenaline.

The question remains how to explain the difference between the action of procaine and curarine. Harvey's (6) evidence that in the superior cervical ganglion, procaine diminishes the output of acetylcholine when the preganglionic fibres are stimulated seems to provide the answer. If this occurs at the neuromuscular junction as well, then the diminished output of acetylcholine and the curarine-like action cover most of the facts.

The curarine-like action alone is sufficient to account for the prevention of paralysis with fast stimulation in the presence of prostigmine (figure 6, C), and for the prevention of paralysis produced by injection of acetylcholine (figure 7, B). The curarine-like action is however not sufficient to explain the prevention of the depression produced by adrenaline (figure 5, A) for adrenaline itself has an action overcoming that of curarine. Moreover, as shown in figure 5, B, procaine and cocaine convert the depression produced by adrenaline in a muscle stimulated at higher rates and injected with prostigmine into an augmentation. This reversal is best explained by a decreased acetylcholine production, so that the stage of excess is never reached in the presence of cocaine or procaine.

## SUMMARY

1 The interaction of procaine or cocaine with prostigmine and adrenaline has been studied on the cat's gastrocnemius stimulated indirectly by single maximal shocks. Procaine and cocaine have an action like that of curarine, but whereas curarine depresses normal contractions more than it depresses those seen after the injection of prostigmine, procaine or cocaine depress those seen after prostigmine very greatly while exerting little action on normal contractions.

2 After the injection of procaine or cocaine the augmentor action of prostigmine is abolished, while after the injection of curarine, the augmentor action of prostigmine persists.

3 Adrenaline, injected after prostigmine, has been shown to augment muscle contractions. Although procaine abolishes the action of prostigmine, it does not abolish the augmentor action of adrenaline injected after prostigmine.

4. If adrenaline is injected after prostigmine, the injection of procaine then depresses the effect of prostigmine little or not at all.

5 While the above effects of procaine may be regarded as like those of curarine, procaine also appears to depress the production of acetylcholine at the neuromuscular junction.

Our thanks are due to Dr. Edith Bülbring for directing this work, and to Mr. H. W. Ling for help with the experiments.

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tendency to go into hypoglycemic crises. However, they never developed permanent diabetes insipidus, showed either a temporary increase of water exchange lasting for 1-3 days postoperatively or no increase at all. The median eminence was not destroyed in these animals, it was still capable of producing antidiuretic hormone.

In contrast to this the neurohypophysectomized dogs developed permanent diabetes insipidus. Within the first 24 hours after the operation they showed polyuria and polydipsia, which became more pronounced on the second day and lasted for 5-7 days. This phase of temporary diabetes insipidus was followed by a return to normal water exchange for 3-6 days (normal interphase), after which permanent diabetes insipidus developed by the 10th-12th postoperative day. The diabetes insipidus persisted from then on over a period of observation of 4

TABLE 2

*Water excretion after ingestion of water and the effect of morphine on water diuresis in hypophysectomized dogs*

All experiments started 17-18 hours after last feeding. Amount of first water 40 cc/kg by stomach. 3 hours later bladder emptied by catheterization and second water (40 cc/kg) given by stomach. Time of morphine administration as in table 1. Injected either subcutaneously (5 mg/kg) or intravenously (2.5 mg/kg).

DOG NUMBER	NUMBER OF EXPTS	DOSE OF MORPHINE SULPHATE	URINE DURING 3 HRS IN % OF SECOND WATER GIVEN AVERAGE (MIN MAX.)	DOG NUMBER	NUMBER OF EXPTS	DOSE OF MORPHINE SULPHATE	URINE DURING 3 HRS IN % OF WATER GIVEN AVERAGE (MIN., MAX.)
		mg/kg				mg/kg	
H 5	5		59 (47, 73)	H 8	6		51 (47, 53)
H 5	2	5, 5	17 (12, 22)	H 8	2	5, 2.5	7 (3, 11)
H 6	6		56 (48, 72)	H 9	5		48 (42, 56)
H 6	2	5, 2.5	15 (10, 20)	H 9	2	5, 2.5	10 (6, 14)
H 7	5		51 (40, 60)	H 10	6		49 (45, 55)
H 7	2	5, 2.5	19 (16, 22)	H 10	2	5, 2.5	8 (4, 12)

years. These animals excreted 3500-6800 cc of urine daily. They had normally functioning anterior pituitary glands, as evidenced by their normal post-absorptive blood sugar levels, their ability to withstand fasting for a period of eight days as well as normal animals and their normal response to insulin. Only two dogs of this group were somewhat more sensitive to insulin than the normal dogs, but even those were by no means as sensitive as the adeno-hypophysectomized animals.

The hypophysectomized animals excrete water much more slowly than the normals, the amount of urine excreted in the 3 hours following the administration of the second dose of water was an average of 52% (table 2) as compared to the average of 90% for normals (table 1). This protracted excretion of water may be due to the diminished blood pressure (Houssay 12) and decreased renal blood flow with resultant diminished glomerular filtration as found by White, Heinbecker and Rolf (13) in adeno-hypophysectomized animals. Morphine produces

a very marked antidiuretic action in these animals (table 2), i.e. in this respect the hypophysectomized animals do not differ from the normal or adrenal inactivated animals

However, entirely different results were obtained with the diabetes insipidus animals. These animals excreted the ingested water much more rapidly than the adeno-hypophysectomized dogs, but not quite as promptly as the normal animals (table 3). The amount of urine excreted in 3 hours, by the 10 diabetes insipidus dogs in 92 experiments following the ingestion of water was an average of 73% as compared to the average of 90% in normal dogs. It should be noted that the figures representing the percentual recoveries of the water administered by stomach in the diabetes insipidus dogs show a greater deviation from the averages than that observed in normal animals. This is quite understandable considering the greatly increased water exchange present in these animals.

As can be seen in table 3 morphine (given 40 minutes after the administration of water by stomach tube) did not inhibit the water diuresis in the diabetes insipidus dogs.

In another series of experiments the water was infused intravenously using the same technique as described above for normal animals. As can be seen in table 3, the water was excreted usually in excess, and morphine, given 15 minutes before the beginning of the water infusion did not inhibit the water diuresis. The diabetes insipidus dogs while under the influence of morphine, excreted in 3 hours an average of 107% of the intravenously administered water in contrast to the average excretion of 14% observed in normal animals under similar conditions.

It is thus clear that morphine has no antidiuretic action in the diabetes insipidus dogs but inhibits water diuresis only if a functioning neurohypophysis is present in the animal in other words the antidiuretic hormone of the neurohypophysis is necessary for the antidiuretic action of morphine.

*The effect of morphine on chloride excretion and saline diuresis* In search of further evidence that the antidiuretic hormone of the pituitary gland is involved in morphine antidiuresis, studies were made of the effect of morphine on urinary chloride concentration and on saline diuresis. It is well established that the antidiuretic hormone of the posterior pituitary gland brings about a percentual and absolute increase in the urinary chloride excretion and also that it either does not inhibit saline diuresis at all or inhibits it to a lesser degree than it does water diuresis depending upon the concentration of sodium chloride solution infused intravenously. These changes are quite characteristic of the antidiuretic hormone of the pituitary gland and if morphine produced similar changes, it would be further strong evidence in favor of the fact that the neurohypophysial antidiuretic hormone is essential to morphine antidiuresis. To a series of normal dogs the first and second doses of water were given in the usual way and the chloride excreted in the urine during the 3 hours following the administration of the second water was determined. The experiments were then repeated with the administration of the usual dose of morphine given as described above. In order to obtain the chlorides quantitatively (especially in the morphine experi-

morphine excreted in every instance only a small fraction of the second water (see table 1). Though it is clear that in these experiments morphine could not have interfered with the absorption of water, an additional series of experiments were carried out in which the second water was administered intravenously (25 cc/kg). Table 1 shows that in the animals which did not receive morphine the intravenously administered water was excreted either quantitatively or in excess. In 6 out of 14 experiments the excreted amount varied between 121 and 142%, in the remainder, the excreted amount was between 88 and 100%. The water was infused into a large vein, slowly, over a period of 30 minutes to minimize the occurrence of hemolysis. In some cases the urine was entirely free

TABLE 1

*Water excretion after ingestion or intravenous infusion of water and the effect of morphine on water diuresis in normal dogs*

All experiments started 17-18 hours after last feeding

NUM BER OF DOGS	NUM BER OF EXPER IMENTS	FIRST WATER		URINE DURING 3 HRS IN % OF FIRST WATER GIVEN AVERAGE (MIN. MAX.)	SECOND WATER		DOSE OF MOR PHINE SULFATE	URINE DURING 3 HRS IN % OF SECOND WATER GIVEN AVERAGE (MIN., MAX.)
		Given by	Amount cc/kg		Given by	Amount cc/kg		
32	84	Stomach	40	88 (66, 113)	Stomach	40		90 (71, 109)
14	14	Stomach	40	90 (71, 109)	Vein	25		111 (88, 142)
24	48	Stomach	40	87 (69, 108)	Stomach	40	5 subcut or 2.5 intrav	12 (1, 28)
14	14	Stomach	40	87 (72, 106)	Vein	25	5 subcut or 2.5 intrav	14 (2, 30)

\* Time of morphine administration 40 minutes after the second dose of water was administered, when this was given by stomach, or 15 minutes before the intravenous infusion of the second dose of water was started. In the former case the urine excreted in the interval between the administration of the second dose of water and the injection of morphine was quantitatively collected and deducted from the total amount of second water in calculating the percentual recovery.

from hemoglobin. However in most cases some degree of hemolysis did occur. The amount of hemoglobin in the urine varied, the urine in the majority of the experiments containing only small amounts. Morphine, which was injected 15 minutes before the infusion of water was started, inhibited the excretion of water in these experiments to precisely the same degree as it did in those experiments in which the second water was given by stomach.

It was thus conclusively established that morphine exerts an inhibiting influence on water diuresis independent of any effect it may have indirectly through its gastro intestinal or thirst-abolishing actions.

With the antidiuretic action of morphine thus clearly demonstrated, experiments were devised to investigate the mechanism of this action.

*The effect of morphine on water diuresis in adrenal-inactivated dogs.* Since it was shown by Bodo, Co Tui and Benaglia (7) that morphine brings about the

liberation of adrenaline from the adrenal glands the possibility that renal ischemia produced by adrenaline might be the immediate cause of the morphine antidiuresis had to be considered. Richards and Plant (8, 9) and Richards and Schmidt (10) have shown that adrenaline in small doses constricts preferentially the efferent arterioles of the kidney and thereby while decreasing the blood flow through the kidney increases the intraglomerular pressure and hence the urine formation. However larger amounts of adrenaline may constrict effectively both the afferent and efferent vessels with resultant diminished blood flow, intraglomerular pressure and urine formation. If morphine inhibits water diuresis by causing renal ischemia we have to assume that adrenaline is liberated in such quantities that it constricts the afferent vessels also.

In order to investigate this possibility the effect of morphine on water diuresis in adrenal inactivated dogs was studied. The adrenal inactivation consisted of removal of the right adrenal gland and denervation and demedullation of the left. For details of the operative technique see Bodo et al (7). Only those animals are included here in which the histological examination of the left adrenal gland removed at autopsy verified the absence of the medullary tissue.

These animals excreted water (second dose) like the normal animals and morphine (given 40 minutes after the second dose of water) inhibited the water diuresis to the same extent as in the normals; therefore the adrenaline liberated by morphine cannot be considered to be the cause of the antidiuresis.

*The effect of morphine on water diuresis in hypophysectomized and in diabetes insipidus dogs.* The next step in the analysis was to study the rôle of the pituitary gland in morphine antidiuresis. For this purpose two types of animals were used. In the first type the entire adenohypophysis (pars distalis, pars intermedia and pars tuberalis) and in addition the infundibular process (neural lobe) was removed in one piece. In the second type a high hypophysial stalk section was carried out with additional hypothalamic injury resulting in loss of function and degeneration of the entire neurohypophysis (infundibular process, infundibular stem and median eminence). The first type we designate as hypophysectomized dogs, the second as diabetes insipidus or neurohypophysectomized dogs. The hypophysectomies were done by the oral approach; the hypophysial stalk sections and hypothalamic injuries were done either by the temporal route or by the oral route. For further details see de Bodo et al (11). The hypophysectomies were considered complete only if the sections of a block including the body of the sphenoid bone, the fibrous tissue occupying the sella turcica and the overlying brain tissue did not contain pituitary cells and the thyroids, adrenals and gonads showed the typical atrophic changes characteristic of animals from which the entire adenohypophysis has been removed. The histological studies were made by Dr David Marine, Director of the Laboratories of Montefiore Hospital, New York City. A detailed report of these studies is now in preparation and will be published in the near future in collaboration with Dr Marine.

The hypophysectomized animals had a low postabsorptive blood sugar level, were sensitive to insulin, were unable to withstand fasting and showed a marked

tendency to go into hypoglycemic crises. However, they never developed permanent diabetes insipidus, showed either a temporary increase of water exchange lasting for 1-3 days postoperatively or no increase at all. The median eminence was not destroyed in these animals, it was still capable of producing antidiuretic hormone.

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As can be seen in table 3 morphine (given 40 minutes after the administration of water by stomach tube) did not inhibit the water diuresis in the diabetes insipidus dogs.

In another series of experiments the water was infused intravenously using the same technique as described above for normal animals. As can be seen in table 3, the water was excreted usually in excess, and morphine, given 15 minutes before the beginning of the water infusion, did not inhibit the water diuresis. The diabetes insipidus dogs while under the influence of morphine excreted in 3 hours an average of 107% of the intravenously administered water in contrast to the average excretion of 14% observed in normal animals under similar conditions.

It is thus clear that morphine has no antidiuretic action in the diabetes insipidus dogs but inhibits water diuresis only if a functioning neurohypophysis is present in the animal, in other words the antidiuretic hormone of the neurohypophysis is necessary for the antidiuretic action of morphine.

*The effect of morphine on chloride excretion and saline diuresis.* In search of further evidence that the antidiuretic hormone of the pituitary gland is involved in morphine antidiuresis, studies were made of the effect of morphine on urinary chloride concentration and on saline diuresis. It is well established that the antidiuretic hormone of the posterior pituitary gland brings about a percentual and absolute increase in the urinary chloride excretion and also that it either does not inhibit saline diuresis at all or inhibits it to a lesser degree than it does water diuresis depending upon the concentration of sodium chloride solution infused intravenously. These changes are quite characteristic of the antidiuretic hormone of the pituitary gland and if morphine produced similar changes, it would be further strong evidence in favor of the fact that the neurohypophysial antidiuretic hormone is essential to morphine antidiuresis. To a series of normal dogs the first and second doses of water were given in the usual way and the chloride excreted in the urine during the 3 hours following the administration of the second water was determined. The experiments were then repeated with the administration of the usual dose of morphine given as described above. In order to obtain the chlorides quantitatively (especially in the morphine experi

ments) after the catheterization the bladder was washed with 250 cc of water. Chlorides were determined by a modified Volhard-Harvey method (Peters and Van Slyke, 14), adding two drops of caprylic alcohol to promote coagulation of the silver chloride and filtering the solution.

TABLE 3

*Water excretion after ingestion or intravenous infusion of water and the effect of morphine on water diuresis in diabetes insipidus dogs*

All experiments started 17-18 hours after last feeding. Time of morphine administration as in table 1. Injected either subcutaneously or intravenously.

DOG NUMBER	A WATER (40 CC./KG.) GIVEN BY STOMACH			B WATER (25 CC./KG.) GIVEN BY VEIN		
	Number of expts	Dose of morphine sulphate	Urine during 3 hrs. in % of water given Average (min max)	Number of expts	Dose of morphine sulphate	Urine during 3 hrs. in % of water given
DI 2	13	5	73 (66, 88)	2	5	194, 135
	3		70 (68, 73)	2		101, 75
DI 6	13	5	84 (65, 146)	2	5	249, 137
	3		88 (87, 89)	2		114, 112
DI 13	13	5	98 (84, 117)	2	5	106, 107
	2		87 (82, 92)	2		125, 79
DI 15	14	5	70 (62, 91)	2	5	128, 130
	2		60 (64, 68)	2		61, 77
DI 17	11	5	102 (84, 133)	2	5	122, 181
	2		81 (77, 85)	2		126, 131
DI 12	7	5	71 (66, 81)	2	5	128, 114
	2		69 (68, 70)	2		101, 97
DI 16	7	5	84 (64, 131)	2	5	132, 122
	2		85 (82, 88)	2		114, 116
DI 18	6	5	78 (65, 96)	2	5	127, 131
	2		75 (74, 76)	2		115, 120
DI 7	5	5	98 (92, 109)	1	5	122
	1		92	1		115
DI 14	3	5	71 (66, 78)	1	5	158
	1		98	1		179

Table 4 shows the changes in chloride excretion induced by morphine. It can be seen that the urine excreted during the period of morphine action contains increased amounts of chlorides—both relatively and absolutely. In other words the changes produced by morphine are similar to those produced by pituitrin. However, it should be emphasized that these changes may not occur if the animal

vomits repeatedly due to morphine and thereby loses chlorides. On a larger number of animals it was observed that no vomiting occurred when morphine was injected intravenously. Therefore the intravenous administration of morphine was used in the experiments in which the urinary chloride excretion was studied.

TABLE 4

*The effect of morphine on urinary chloride excretion in normal dogs*

All experiments started 17-18 hours after last feeding. Amount of first water 40 cc./kg by stomach. 3 hours later bladder emptied by catheterization, and second water (40 cc./kg given by stomach. Time of morphine administration as in table 1. Injected intravenously (2.5 mg./kg.)

DOG NUMBER	CHLORIDE EXCRETED IN URINE DURING 3 HOURS FOLLOWING ADMINISTRATION OF SECOND WATER			
	A. Control expts.		B. Morphine expts.	
	mg. %	mg./hour	mg. %	mg./hour
N 1	2.34	3.94	166.0	9.45
N 2	5.62	6.92	172.3	12.83
N 3	4.20	8.15	221.4	21.30
N 4	4.39	8.11	195.0	18.21
N 5	3.54	6.54	240.5	11.35
N 6	4.53	8.81	232.3	12.65
N 7	2.42	5.42	85.67	16.41

TABLE 5

*The effect of posterior pituitary extract on water exchange in diabetes insipidus dogs*

All experiments started 17-18 hours after last feeding. The bladder of each animal was emptied by catheterization. Animals allowed to drink ad lib. Pituitrin (Parke Davis & Co.) injected subcutaneously. Dose 100-200 millunits.

DOG NUMBER	WATER EXCHANGE DURING 6 HRS.			
	A. Control experiments		B. Pituitrin experiments	
	Water drunk	Urine output	Water drunk	Urine output
	cc.	cc.	cc.	cc.
DI 2	960	830	220	330
DI 6	1020	956	190	290
DI 13	1060	920	210	336
DI 15	1240	1090	150	185
DI 17	1180	1025	220	207
DI 12	990	850	230	190

Each figure represents the average of 5-6 experiments.

The effect of morphine on saline diuresis is also similar to that of pituitrin. Depending upon the concentration of sodium chloride solution infused intravenously it either does not inhibit saline diuresis at all or inhibits it to a lesser degree than it does water diuresis.

*The effect of morphine plus injected pituitrin on water diuresis in diabetes insipidus*

*dogs* In evaluating the rôle of the antidiuretic hormone of the pituitary gland in morphine antidiuresis two possibilities must be considered. Morphine may potentiate the antidiuretic action of the circulating neurohypophysial hormone and thereby inhibit water diuresis or it may stimulate the production or increase the liberation of the antidiuretic hormone from the neurohypophysis.

In order to test the first possibility the following experiments were carried out. In a series of experiments diabetes insipidus dogs were given varying doses of posterior pituitary extract until that dose was found which would lower their water exchange to a more or less normal level without however preventing the quantitative excretion of water administered by stomach tube (first and second

TABLE 6

*The effect of posterior pituitary extract and morphine plus posterior pituitary extract on water diuresis in diabetes insipidus dogs*

All experiments started 17-18 hours after last feeding. The bladder of each animal was emptied by catheterization. 100-200 milliunits of Pituitrin (Parke, Davis & Co.) injected subcutaneously. One hour later 40 cc/kg water given by stomach tube. When morphine was given, it was injected intravenously (5 mg/kg) 40 minutes after the administration of the water by stomach tube.\*

DOG NUMBER	URINE DURING 3 HRS. IN % OF WATER GIVEN BY STOMACH TUBE†	
	A Pituitrin	B Morphine + pituitrin
DI 2	71	69
DI 6	97	88
DI 13	98	91
DI 15	66	61
DI 17	74	76
DI 12	85	87
Average	82	79

\* The urine excreted in the interval between the administration of water and the injection of morphine was quantitatively collected and deducted from the total amount of water in calculating the percentual recovery.

† Each figure represents the average of 3-4 experiments.

doses) As can be seen in table 5 100-200 milliunits of pituitrin given subcutaneously lowered the water exchange of the diabetes insipidus dogs from the characteristically high level to a relatively normal level which persisted throughout the period of observation of 6 hours. When this amount of pituitrin was given and one hour later 40 cc/kg water was administered by stomach tube, 82% of the water was excreted on the average (table 6). When the same experiment was repeated and in addition morphine was injected intravenously 40 minutes after the administration of the water by stomach, the excretion of water was about the same as without the drug (table 6). If morphine exerted its antidiuretic action in the normal animal by potentiating the effect of the circulating antidiuretic hormone of the posterior pituitary gland, it should have reduced the

excretion of the administered water in these experiments as it did in the normal animals. Since it had no such effect, it can be concluded that morphine does not exert its antidiuretic action in the normal animal by potentiating the circulating posterior pituitary hormone but by increasing the secretion or liberation of the hormone. This might be a "release phenomenon" or morphine may actually stimulate the hypothalamico-hypophysial system either by acting on the supra-optic or paraventricular nuclei or on the pituitrin secreting cells themselves.

**DISCUSSION AND CONCLUSIONS** There are still a few points that need further elucidation. It is well known that many forms of smooth muscle are stimulated to increased contraction and tone under the influence of morphine and the other phenanthrene alkaloids. The bladder and ureter might be included in this group. It was shown by Edmunds and Roth (15) that morphine causes an increase in tone and in amplitude of contractions of the cat's bladder whether in situ or isolated, which was abolished by atropine. Van Duzer et al. (16) showed that the detrusor muscle of the bladder is stimulated by morphine. Macht (17) found that morphine increases the contractions and produces a greater tonicity of the ureter whether isolated (pig human) or in situ (rabbit). Ockerblad et al. (18) and Carroll and Zingale (19) found the same for the intact human ureter and also showed that these contractions induced by morphine were abolished by atropine.

There is then sufficient evidence to show that morphine increases the tone and contractions of the ureter and bladder either in situ or when isolated and that this effect is abolished by atropine. This effect of morphine is given in the textbooks as the explanation for the urinary retention observed in animals and humans and for the inhibition of water diuresis. However this explanation is invalid. The action of morphine on the bladder and ureter cannot account for its antidiuretic action in normal animals first because morphine must act equally on the bladder and ureters of the diabetes insipidus and normal animals since it acts even on the isolated organs, but despite this action morphine produces no antidiuretic effect in diabetes insipidus animals. Secondly, atropine abolished the action of morphine on the ureter and bladder but the antidiuretic action of morphine is not influenced by the presence or absence of effective doses of atropine. The evidence for this will be presented in another paper.

Another question that has to be dealt with here is the possible effect of morphine upon the composition of blood plasma as a cause of its antidiuretic action. Some anesthetics (barbiturates) in full anesthetic doses cause a transient hemodilution often followed by hemoconcentration. If a similar hemoconcentration resulted from morphine administration, its antidiuretic effect might be attributed to this effect. However morphine even in much larger doses than used in our experiments caused only negligible changes in hematocrit readings (Green et al. 20). Thus hemoconcentration and oligemia cannot be considered as the cause of morphine antidiuresis.

The experiments presented in this paper lead us to the conclusion that morphine inhibits water diuresis by an action upon the hypothalamico-hypophysial system. It is of interest to attempt to investigate the more intimate mechanism

of this action M Pickford (21) has shown that acetylcholine inhibits water diuresis in the normal dog, that this inhibition is accompanied by a percentual and absolute rise in the urinary chlorides and that the antidiuretic effect does not occur after removal of the posterior pituitary body Bernheim (22, 23) presented evidence that morphine inhibits cholinesterase, Slaughter et al (24, 25) found that eserine potentiates some of the effects of morphine In view of these experimental findings the concept suggests itself that acetylcholine may be involved in the antidiuretic action of morphine This will be discussed in a further publication

#### SUMMARY

1 When normal dogs in the postabsorptive state and in water equilibrium are given water, either by stomach (40 cc/kg) or by slow intravenous infusion (25 cc/kg), they excrete it practically quantitatively within three hours

2 The water administered by stomach (40 cc/kg) is completely absorbed from the gastro-intestinal tract in 40 minutes

3 Morphine, given 40 minutes after the administration of water by stomach or 15 minutes before the intravenous infusion of water is started, inhibits water diuresis very markedly (excreted amount 2-15%)

4 The inhibition of water diuresis due to morphine is accompanied by a percentual and absolute increase in chloride excretion

5 Morphine either does not inhibit saline diuresis or inhibits it to a lesser degree than it does water diuresis

6 The adrenaline liberated due to morphine is not involved in the morphine antidiuresis

7 Morphine does not inhibit water diuresis in diabetes insipidus dogs It inhibits water diuresis only if a functioning neurohypophysis is present in the animal, the antidiuretic hormone of the neurohypophysis is necessary for the antidiuretic action of morphine

8 Morphine does not potentiate the antidiuretic action of the circulating posterior pituitary hormone

9 Morphine brings about the liberation of the antidiuretic hormone by acting on the hypothalamico hypophysial system

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# THE EFFECT OF ASCORBIC ACID ON THE ACUTE TOXICITY OF SULFANILAMIDE FOR GUINEA PIGS

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The toxic effect of sulfanilamide in humans appears to be reduced by ascorbic acid (3, 4). In order to determine the relationship between sulfanilamide and vitamin C in another species unable to synthesize C, the guinea pig was chosen as an ideal animal. This paper is concerned with the effect of ascorbic acid on the toxicity of sulfanilamide in guinea pigs.

**METHODS** The amount of sulfanilamide which kills fifty per cent of the animals ( $LD_{50}$ ) was determined according to the methods of Gaddum (6) and of Bliss (1, 2).

The sulfanilamide was administered by stomach tube as a twenty per cent suspension in water, and the dose was calculated in terms of grams per kilogram of body weight. All animals were observed for seven days.

Guinea pigs used in these experiments belonged to both sexes and weighed approximately 300 grams. All animals were observed for at least three days before being used.

The  $LD_{50}$  was determined for both fasting and non-fasting guinea pigs. Non-fasting animals were fed on a diet of mixed grains, cod liver oil (U S P XI), brewer's yeast (Fleischman), and greens *ad lib*. Food was removed from the cages of fasting animals about fifteen hours before treatment was begun.

The non-fasting animals were divided into two groups: a) normal and b) high vitamin C. The fasting animals were divided into three groups: a) normal, b) high vitamin C, and c) depleted.

Ascorbic acid (Merck's Cebione) was administered intraperitoneally in aqueous solution freshly prepared prior to each series of injections, and the pH was adjusted to approximately 7.4.

Guinea pigs receiving ascorbic acid were injected several times during the experiment to maintain a high blood vitamin C. Some were injected with 10.0 mgm ascorbic acid  $\frac{1}{2}$  hour prior to the administration of the single dose of sulfanilamide, 10.0 mgm 1 hour after, and 10.0 mgm again  $2\frac{1}{2}$  hours after the sulfanilamide had been given. Others were given, in addition to these three doses, a fourth injection of 10.0 mgm on the day of the sulfanilamide dosage and three 10.0 mgm injections daily for two successive days. As there was no significant difference between these two series of guinea pigs receiving ascorbic acid, they were combined and are referred to as the high vitamin C animals.

Animals were depleted by depriving them of greens for ten days.

**RESULTS** The  $LD_{50}$  of sulfanilamide for normal, non fasting guinea pigs is 3.13 grams per kg, for fasting, normal guinea pigs, 2.16 grams per kg.

Neither massive repeated injections of ascorbic acid nor ten day depletion of vitamin C affects the acute toxicity of sulfanilamide for guinea pigs.

TABLE 1

*LD<sub>50</sub> s of sulfanilamide for fasting and non fasting guinea pigs in different states of vitamin C nutrition*

NON-FASTING GUINEA PIGS (MAY-JULY 1940)			FASTING GUINEA PIGS (AUGUST-NOVEMBER, 1940)		
Dose	Mortality	Per cent mortality	Dose	Mortality	Per cent mortality
Normal animals					
<i>gms per kg</i>			<i>gms per kg</i>		
3.90	3/4	75	3.87	10/11	91
3.66	1/4	25	3.35	13/14	93
3.45	4/7	57	2.92	12/14	86
3.38	19/23	83	2.58	24/31	77
3.31	3/8	38	2.41	16/20	80
3.21	1/4	25	2.24	4/11	36
			1.90	2/11	18
LD <sub>50</sub> = 3.13 (2.78-3.51)			LD <sub>50</sub> = 2.16 (1.97-2.36)		
High vitamin C					
3.45	4/5	80	3.87	15/16	94
3.38	15/30	50	3.35	16/19	84
			2.92	17/19	89
			2.24	4/22	18
			1.90	7/22	32
LD <sub>50</sub> = 3.29 (2.90-3.75)			LD <sub>50</sub> = 2.40 (2.19-2.63)		
Vitamin C depleted					
			2.58	6/7	86
			2.24	8/12	67
			1.90	4/13	31
			LD <sub>50</sub> = 2.05 (1.78-2.35)		

Since slopes (b) for these groups do not differ significantly all LD<sub>50</sub> s were calculated with a composite 'b' which is equal to  $7.937 \pm 1.154$

Figures in parentheses following the LD<sub>50</sub> s refer to Range of LD<sub>50</sub> 95 times in 100

TABLE 2

*Significant difference of means between LD<sub>50</sub> s of sulfanilamide for fasting and non-fasting guinea pigs in different states of vitamin C nutrition*

	NON-FASTING		FASTING		
	Normals	Vitamin C	Normals	Vitamin C	Vitamin C depleted
Non Fasting					
Normals		0.56	4.46	3.28	4.40
Vitamin C	0.56		5.37	3.62	4.67
Fasting					
Normals	4.46	5.37		1.51	0.61
Vitamin C	3.28	3.62	1.51		1.88
Vitamin C depleted	4.40	4.67	0.61	1.88	

Any number greater than 2.75 indicates that the difference between the compared LD<sub>50</sub> s is significant (Probability = .01 ( $t = 2.75$ ) for 30 or more animals (5))

## SUMMARY

1 The  $LD_{50}$  of sulfanilamide for fasting guinea pigs is significantly less than that for non fasting animals

2 Vitamin C has no influence on the acute toxicity of sulfanilamide for guinea pigs

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# COMPARISON OF THE ACTION OF 1 ETHYL THEOBROMINE AND CAFFEINE IN ANIMALS AND MAN

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Although 1-ethyl theobromine has long been known in chemical literature, study of its pharmacologic action has been meager indeed. The compound was first prepared by van der Slooten (1) in 1897 but nothing of its pharmacology was recorded until 1905 by Bergell and Richter (2). The latter authors concluded that 1-ethyl theobromine possessed a diuretic action about equal to caffeine, no mention of other actions being included in their report. Subsequently, only different methods for preparation of this substance were published.

The various xanthines are known to have similar effects on different functions of the body, but these actions vary in degree. Thus, theophylline although superior as a diuretic is less stimulating to respiration than caffeine. Stimulation of the central nervous system by caffeine has long been known and exhaustively studied in all aspects. In this respect caffeine may be superior to benzedrine (3) but the diuretic action of the former is considered by some to be a slight disadvantage. Substitution of the methyl group in the 1 position of the caffeine molecule with other alkyl groups might enhance the stimulating effect on the nervous system while leaving the diuretic action unchanged or even decreased. In the present report the pharmacology of caffeine is compared to its closest substitution product 1-ethyl theobromine. The latter compound was generously supplied by Dr. L. P. Hyndes of the Monsanto Chemical Company, St. Louis.

**GENERAL PROPERTIES.** 1-Ethyl theobromine is a white crystalline substance, soluble in water and alcohol, slightly soluble in ether, and readily soluble in chloroform or acid water. It has a melting point of 165–166°C. The structural formula of the compound is the same as that of caffeine except an ethyl group is substituted for the methyl radical in the 1-position.

**ACTION ON THE CENTRAL NERVOUS SYSTEM.** *Convulsive action in mice.* Following injection into the tail vein of starved albino mice, 1-ethyl theobromine produced convulsions similar to those of caffeine—that is, mainly tetanic in nature. These convulsions were about 1 minute in duration and if death did not result, the animals recovered rapidly. A period of increased motor activity (restlessness) was noticeable for 1 or 2 hours thereafter. Table 1 shows the results of a determination of the median convulsive dose as calculated by the method of Bliss (4). Caffeine was tested at the same time for comparison. 1-Ethyl theobromine appeared to be about 50 per cent more stimulating to the central nervous system than caffeine as judged from dosage.

*Studies in human beings.* Six healthy young adults acted as subjects to test the effect of 1-ethyl theobromine on finger tapping rate and critical fusion fre-

quency of flicker According to Simonson and Enzer (5, 6), finger-tapping rate and critical fusion frequency measure the function of motor centers and visual centers, respectively These tests also have been employed by Steinhaus and Kelso (7) in studies of efficiency of human beings Furthermore, Simonson and associates (8, 9) have shown that amphetamine and pervitin produce increased scores in these tests, while alcohol (10) has a depressant effect Thus, it appears that these procedures measure the degree of efficiency of the central nervous system

The 6 subjects were trained for more than a month before the actual test period The tests were run on 9 consecutive working days (Monday to Friday, inclusive) The subject was told he would receive either 1-ethyl theobromine, caffeine, or a placebo, but did not know specifically which compound was to be given The drugs were administered orally in gelatin capsules to avoid discovery

TABLE 1

*The median convulsive doses of 1-ethyl theobromine and caffeine by intravenous injection into mice*

DRUG	DOSE	NUMBER CONVULSED/ NUMBER USED	CD <sub>50</sub> $\pm$ S.E.
	mg per kg		mg per kg
1 Ethyl Theobromine	50	3/10	54.1 $\pm$ 2.0
	56	6/10	
	62	8/10	
	80	10/10	
Caffeine	50	0/5	84.4 $\pm$ 4.9
	80	4/10	
	100	8/10	
	125	10/10	

by taste The dosage used was 200 mg in each case After the subject took the capsule at 8 00 a.m., hourly tests were run throughout the day until 4 00 p.m., inclusive Subjects were not allowed to have coffee, tea, or other caffeine-containing beverages on test days All individuals continued their usual work in the laboratories during the experimental period

The results of the finger-tapping tests are shown in table 2 Both 1-ethyl theobromine and caffeine produced consistently greater scores The differences between treatments (placebo, 1-ethyl theobromine, and caffeine) were highly significant when submitted to an analysis of variance (11) Furthermore, caffeine was somewhat more effective than 1-ethyl theobromine in stimulating the rate of finger-tapping

In table 3 are seen the effects of these compounds on critical fusion frequency of flicker Here the mean values obtained with both 1-ethyl theobromine and caffeine were always greater than those of the controls Statistical analysis also showed these differences to be highly significant Caffeine was found again to be more stimulating than 1-ethyl theobromine in equal dosage

During the 9-day test period the subjects each day were given a questionnaire to answer. This consisted of three questions concerning (1) subjective feelings the same day (2) falling asleep on going to bed and (3) subjective feelings the next morning. Of 18 subject-days on each compound, stimulation occurred 4 times while depression was noted 3 times with placebo. 1-ethyl theobromine was followed by stimulation 5 times and depression twice while on 6 occasions stimulation was noted after caffeine. Insomnia resulted 4 times, 6 times and 5 times

TABLE 2

*The influence of 1-ethyl theobromine and caffeine on rate of finger-tapping*  
Oral administration

DRUG	DOSE mg per kg	TIME OF DAY							
		9 a.m.	10 a.m.	11 a.m.	12 noon	1 p.m.	2 p.m.	3 p.m.	4 p.m.
Placebo		457†	440	451	450	445	444	440	448
1 Ethyl Theobromine	200	461	461	461	453	457	459	453	450
Caffeine	200	465	460	459	456	458	464	450	450

The drug or placebo was taken at 8 a.m.

† Each figure is the average tapping rate for 1 minute taken from 18 tests made on 3 separate days and 6 subjects.

TABLE 3

*The effect of 1-ethyl theobromine and caffeine on critical fusion frequency of flicker*

DRUG	O. AL. DOSE mg per kg	TIME OF DAY							
		9 a.m.	10 a.m.	11 a.m.	12 noon	1 p.m.	2 p.m.	3 p.m.	4 p.m.
Placebo		40.1†	49.1	48.7	48.8	48.8	40.0	40.0	48.8
1 Ethyl Theobromine	200	49.4	49.2	49.1	49.1	49.4	49.3	49.4	49.3
Caffeine	200	49.7	49.9	49.6	49.8	49.0	49.9	49.8	49.6

Placebo or drug was taken at 8 a.m.

† All values are represented as the number of flickers per second averaged from 18 tests on 6 subjects for 3 separate days.

following placebo, 1-ethyl theobromine and caffeine, respectively. The subjects felt tired the morning after 5 times with placebo, 5 times following 1-ethyl theobromine and twice after caffeine. It is thus quite obvious that no consistent subjective effects were obtained. Furthermore, a dose of 200 mg of 1-ethyl theobromine taken in the morning was not apt to cause insomnia the same night in most individuals.

*Respiratory stimulation in dogs.* The action of caffeine as a respiratory stimulant is well established. Consequently it was appropriate to study 1-ethyl theobromine in this respect. Dogs were anesthetized with sodium phenobarbital. Blood pressure and respiration were recorded with the usual methods. Further

more, a tracheal cannula was inserted and connected to a double valve. The dog was allowed to inspire only fresh air, while all expired air flowed through a modified Pavlov automatic, electro-magnetic clamp (12) into 1 of 2 small spirometers. The apparatus was so constructed that when one spirometer became filled, the Pavlov clamp immediately directed the flow of expired air into the other spirometer. While 1 spirometer was filling, the other spirometer was being evacuated. Each filling of a spirometer was automatically recorded by a signal magnet. This arrangement allowed a quantitative and continuous analysis of respiratory volume, which was necessary in comparing the action of 2 closely related compounds. The tambour method of recording respiration is only semi-quantitative.

Once the dog and apparatus were connected, repeated small doses of sodium phenobarbital were given until respiration was greatly depressed. The experi-

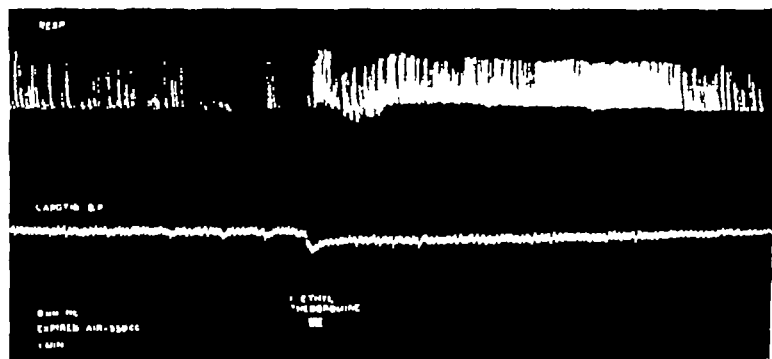


FIG 1 RESPIRATORY STIMULATION PRODUCED BY 1 ETHYL THEOBROMINE

A female dog, weight 5.0 kg., was anesthetized with 150 mg per kg of sodium phenobarbital intravenously. An additional 40 mg per kg of the barbiturate was given, resulting in considerable respiratory depression. The effect of administering 1-ethyl theobromine, 10 mg per kg intravenously, is shown. Note the quantitative increase in volume of expired air.

ment was then allowed to run for 60-90 minutes without manipulation. During this control period, respiration was highly regular and recovery from anesthesia was not apparent. Intravenous injection of either 1-ethyl theobromine or caffeine was made at the end of the control period. A dose of 10 mg per kg was used in all tests. By this procedure, a total of 10 dogs was studied. Only 1 drug was given to each animal in order to avoid any conflict of action. A record of the effect of 1-ethyl theobromine is shown in figure 1. In table 4 it is obvious that caffeine produced less respiratory stimulation than 1-ethyl theobromine.

**DIURETIC ACTION.** *Effects in unanesthetized dogs.* Six female dogs were trained to lie quietly on a table following insertion of an in-lying catheter. On test days 1 hour previous to catheterization, 500 cc of 0.9 per cent sodium chloride solution was administered by stomach tube since the urine output was otherwise too irregular and too small. This dose was equivalent to approximately 50 cc per kg. Lipschitz and associates (13) used 25 cc per kg of isotonic sodium chloride solution in their assays of diuretics in rats. Isotonic saline is known to have little or

no diuretic action of its own when given orally (14). Animals were starved 18 hours prior to the actual test period. Following catheterization, the urinary bladder was emptied at 15-minute intervals. After 45-60 minutes when the urine output was quite regular, an intravenous injection of 10 mg per kg of either 1-ethyl theobromine or caffeine was made. The bladder was emptied every

TABLE 4

*Comparison of the respiratory stimulating actions of 1-ethyl theobromine and caffeine by intravenous injection into dogs*

DRUG AND DOSE	RESPIRATORY VOLUME DURING 30-MINUTE CONTROL PERIOD	RESPIRATORY VOLUME DURING 30 MINUTES FOLLOWING DRUG	PER CENT INCREASE OVER CONTROL	AVERAGE PER CENT INCREASE OVER CONTROL
10 mg per kg.				
1 Ethyl Theobromine	2 56 4 18 3 58 4 40 2 93	4 95* 5 32 4 40 4 95 3 94	93 4 28 8 22 9 12 5 34 4	33 4
Caffeine	3 30 2 93 3 94 3 76 2 93	5 41 2 48 4 40 3 76 3 48	63 9 -15 4 11 7 0 0 18 8	15 8

All figures in these columns represent volume of expired air in liters per 5 minutes

TABLE 5

*The diuretic action of 1-ethyl theobromine and caffeine by intravenous injection into trained unanesthetized dogs*

DOG	TOTAL OUTPUT OF URINE FOR 90 MINUTES FOLLOWING ADMINISTRATION OF DRUG		
	Noose	1 Ethyl Theobromine	Caffeine*
	cc.	cc.	cc.
1	85 3	86 0	198 0
2	41 0	143 0	114 0
3	82 5	111 5	82 0
4	52 0	171 0	47 5
5	83 0	100 0	84 0
6	26 5	72 5	57 5
Mean	62 5	114 0	97 2

Dose was 10 mg per kg in each case

15 minutes for 1½ hours thereafter. In table 5, it is seen that 1-ethyl theobromine produced greater diuresis than caffeine. The value for the former was statistically significant in comparison with untreated animals. In the case of caffeine, the increased urine output was not quite significant by statistical analysis. It appeared that in dogs 1-ethyl theobromine was a stronger diuretic than caffeine.

*Diuretic action in human beings* 1 Ethyl theobromine and caffeine were

tested for diuretic action in 6 healthy young adults. The following procedure was used, which is similar to that used by Keith (15) except that weighed diets were not used. Subjects were instructed to eat their regular diets and to avoid unusual foods. Furthermore, all salty foods were prohibited and a minimum amount of salt was to be added as seasoning. Water intake was limited to 1500 cc per day, no caffeine-containing beverages being permitted. These individuals were all laboratory workers and continued their usual duties during the test days. The urinary bladder was emptied at 8 00 a.m. Immediately afterwards a capsule containing 200 mg of one or the other drug or a placebo was taken orally. Thereafter, until 4 00 p.m. all urine specimens were collected and measured. The bladder was again emptied at 4 00 p.m. and the total urine output recorded for the day's test period. This procedure was followed for 9 consecutive working days. Each drug and placebo were tested on 3 separate days. The results of

TABLE 6

*The influence of 1-ethyl theobromine and caffeine on urine output in human beings  
Oral administration*

SUBJECT	TOTAL URINE OUTPUT FOR THREE TEST PERIODS		
	Placebo	1 Ethyl Theobromine	Caffeine
O M	1678	1615	1717
A O	1415	1605	1430
O R	1780	3045	2245
W W	730	2035	1315
N M	1255	2190	1470
M E	896	1373	1152
Mean daily output	431	659	518

\* Dosage was 200 mg

this diuretic study are shown in table 6. 1-Ethyl theobromine produced somewhat greater diuresis than caffeine. Statistical analysis revealed a highly significant difference between the 3 treatments.

**TOXICITY STUDIES** *Acute toxicity in rats and mice* The median lethal dose of 1-ethyl theobromine was determined in starved albino rats and starved albino mice. Both intravenous and oral routes of administration were used in rats, while mice received the drug by vein only. Calculations were made by the method of Bliss (4). The acute toxicity of caffeine was obtained simultaneously for comparison. Tabulation of results is found in table 7. Caffeine was about 30-50 per cent less toxic than 1-ethyl theobromine by this procedure.

*Feeding experiments on rats* Employing the method of Anderson, Henderson, and Chen (16), young rats weighing about 100 gm were fed a standard diet to which 1-ethyl theobromine was added. Six groups of 5 animals each were used, each group receiving a different percentage of 1-ethyl theobromine in the food. The percentages ranged from 0.02-0.5. A record was kept of the gain in weight

TABLE 7

The acute median lethal doses of 1-ethyl theobromine and caffeine in albino rats and albino mice

ANIMAL	DRUG	ROUTE OF ADMINISTRATION	DOSE	NUMBER DEAD/NUMBER TESTED	LD <sub>50</sub> in g./kg.
Rat	1 Ethyl Theobromine	Oral	mg per kg		
			125	0/10	176 ± 10.3
			180	5/10	
			200	8/10	
			250	12/18	
			300	10/10	
	Caffeine	Oral	200	2/9	233 ± 14.0
			250	7/10	
			300	8/10	
			365	10/10	
	1 Ethyl Theobromine	Intravenous	56	0/8	73.6 ± 3.67
			62.5	4/10	
			70	5/10	
			80	6/10	
			90	7/10	
			110	3/3	
	Caffeine	Intravenous	100	2/10	104.8 ± 1.87
			110	8/10	
			125	9/9	
Mouse	1 Ethyl Theobromine	Intravenous	50	2/10	61.04 ± 2.41
			56	3/10	
			62	4/10	
			80	10/10	
	Caffeine	Intravenous	50	0/5	100.9 ± 6.67
			80	2/10	
			100	5/10	
			125	8/10	

TABLE 8

The effect on growth of feeding 1-ethyl theobromine and caffeine to young rats for 28 days

DRUG	CONCENTRATION OF DRUG IN FOOD	TOTAL WEIGHT GAIN OF 8 RATS
1 Ethyl Theobromine	per cent	gms.
	0.020	477
	0.035	343
	0.070	408
	0.100	348
	0.300	216
	0.500	136
Caffeine	0.020	511
	0.035	406
	0.070	296
	0.100	436
	0.300	262
	0.500	54

One rat died on the fifteenth day of the test.

of each animal. This regime was maintained for 28 days, at the end of which time all animals were sacrificed. An identical procedure was carried out with caffeine for comparison. In table 8 are shown the results. Statistically, there was no difference in the 2 treatments. There was little or no depression of growth with concentrations of either compound up to and including 0.1 per cent. Larger amounts of either drug inhibited weight gain somewhat. One rat died the fifteenth day of the test period, this death occurring in the group fed the greatest percentage of 1-ethyl theobromine.

Post-mortem examination was made of all rats which received the 0.5 per cent concentration of 1-ethyl theobromine or caffeine. No gross or microscopic damage was found in the animals which were given 1-ethyl theobromine, with one exception. The animal which died after 15 days on the diet showed pathological changes associated with emaciation. Of the 5 rats on caffeine, 3 were normal, 1 revealed slight pulmonary edema and congestion, and in the other animal hydronephrosis of the right kidney was noted but no stones were seen.

#### CONCLUSIONS

1 1-Ethyl theobromine in sufficient doses produced stimulation of the central nervous system as evidenced by (a) convulsions in mice, (b) increase in both critical fusion frequency of flicker and finger-tapping rate in adult human beings, and (c) augmentation of respiration in dogs deeply anesthetized with sodium phenobarbital.

2 1-Ethyl theobromine appeared to be less potent than caffeine on the nervous system of human beings, but the former was more convulsant in mice and was a stronger respiratory stimulant in dogs.

3 No untoward effects were noted from an oral dose of 200 mg. of 1-ethyl theobromine taken by 6 adults each for 3 separate days.

4 1-Ethyl theobromine had a diuretic action in human beings and dogs, this action being stronger than that of caffeine.

5 In mice and rats, the acute toxicity of caffeine, orally or intravenously, was 30-50 per cent less than that of 1-ethyl theobromine.

6 Prolonged administration of 1-ethyl theobromine in growing rats' food did not inhibit growth even when concentrations as high as 0.1 per cent were used. Greater amounts depressed growth, but little or no pathological changes occurred with quantities as great as 0.5 per cent of the food. No difference from caffeine was noted.

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# COMPARISON OF ESTERS OF STROPHANTHIDIN IN CATS

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Esters of strophanthidin, the benzoate and *p* bromobenzoate, were first synthesized by Windaus and Hermanns (1) and Jacobs and Heidelberger (2). They are the reaction products of the secondary hydroxyl group on carbon atom 3 of the aglycone, and of benzoic and *p*-bromobenzoic acids. Other esters have been prepared and studied pharmacologically (3, 4, 5, 6). In previous communications (7, 8), we reported the potency in cats of strophanthidin-3-acetate, -propionate, -*n*-butyrate, and -benzoate.

The present study deals with the preparation of thirteen additional esters and the determination of their potency in cats. The names are listed in table 1, with the exception of strophanthidin-3-*p*-nitrobenzoate which is not included for reasons to be given below. Six of them are new, namely, strophanthidin-3-dichloroacetate, - $\alpha$  chloropropionate, -pelargonate, -laurate, -myristate, and -palmitate. These products can be obtained by allowing strophanthidin to react with the acid chloride or acid anhydride in the presence of a catalyst such as pyridine or dimethyl aniline, in an organic solvent such as chloroform. The diethylaminoacetate was prepared by the method of Küssner (5).

All the esters were purified by repeated crystallization from methanol. The melting points are recorded in table 1. Strophanthidin-3-*p*-nitrobenzoate melted at 256–258°C (corrected). The figures for the *n*-caproate and *p*-nitrobenzoate differ considerably from those of Neumann (3). No explanation can be offered for this deviation at the present time except that the rate of heating in our determinations was moderate. The results of combustion analyses were all within the experimental limits customarily allowed in organic synthesis, namely,  $\pm 0.2\%$ .

For cat experiments, stock solutions of 1:1000 in 85–90% ethanol by volume were made with strophanthidin-3-dichloroacetate, -diethylaminoacetate, - $\alpha$ -chloropropionate, -*iso*-butyrate, -*n* caproate, and -*iso*-caproate. Dilutions of 1:50,000–1:25,000, depending on the potency of the product, were employed for intravenous injection until death occurred in the same manner as previously reported (9). The rate of injection was 1 cc per minute. Stock solutions of strophanthidin-3 chloroacetate and -pelargonate were 1:1000 in 95% ethanol, those of -laurate and -palmitate, 1:500 in 85–90% ethanol, and those of -nicotinate and -myristate, 1:500 in absolute ethanol. These six compounds were injected intravenously without dilution at the rate of 0.08–0.04 cc per minute, depending on the potency of the compound, followed by 1 cc of saline by means of a 3-way stopcock—a procedure previously adopted in this laboratory (10). In no instance was the volume of ethanol so great as to be responsible for the death of the animal. Besides, the amount of ether administered gradually diminished as the experiment proceeded in order to avoid excessively deep anesthesia. Strophanth-

TABLE 1

*Toxicity determination of esters of strophanthidin in cats*

STROPHANTHIDIN-3-	MELTING POINT	SEX OF CAT	BODY WEIGHT	DOSE REQUIRED TO KILL
	°C		kg	g per kg
chloroacetate	226.0-227.0	F	1.950	943.6
		F	2.878	889.5
		F	2.033	885.7
		M	2.473	1423.4
		F	2.212	1247.7
dichloroacetate	183.0-184.0	F	1.787	323.3
		F	1.815	441.9
		F	1.787	300.5
		F	1.884	206.4
		F	2.189	373.7
		F	2.436	109.5
		F	2.423	831.0
		F	2.394	422.7
		F	2.203	178.8
		M	2.084	212.1
diethylaminoacetate	192.5-193.5	F	2.534	99.1
		F	1.811	164.5
		F	1.891	173.5
		F	2.687	320.8
		F	1.883	293.1
		M	2.712	153.6
		F	1.927	184.7
		F	2.783	237.5
		M	2.341	358.8
		F	2.026	234.0
$\alpha$ -chloropropionate	158.0-160.0	F	1.818	844.9
		F	1.886	729.6
		M	1.838	1005.4
		F	1.884	297.2
		F	2.169	466.6
iso-butyrate	227.5-228.5	F	1.932	691.5
		M	1.934	575.0
		M	1.901	192.5
		F	1.817	464.5
		F	1.869	814.3
		F	1.874	804.7
$n$ -caproate	177.0-178.0	M	2.907	258.7
		F	1.862	304.5
		M	1.917	173.2
		F	2.118	201.1
		F	1.853	169.5

TABLE 1—*Continued*

STROPHANTHIDIN 3-	MELTING POINT	SEX OF CAT	BODY WEIGHT	DOSE REQUIRED TO KILL
	C		kg	μg per kg
130 capronate	180.0-181.0	F	2.578	215.7
		M	1.980	259.6
		F	1.815	420.9
		F	2.061	286.3
		M	1.915	437.1
nicotinate	253.0-253.5	M	1.960	1143.0
		F	2.047	977.0
		F	1.800	1244.0
		F	1.940	618.6
		F	2.115	983.4
pelargonate	165.0-166.0	F	1.932	414.1
		F	2.410	315.4
		F	2.502	405.9
		F	2.282	525.9
		M	2.609	490.6
laurate	163.0-164.0	M	1.975	1215.2
		F	1.910	816.7
		M	1.955	491.0
		M	2.065	445.5
		F	2.075	694.0
myristate	159.5-160.5	F	1.840	1565.2
		F	2.000	1040.0
		F	2.570	684.8
		F	1.828	1006.6
		F	2.527	823.1
palmitate	156.5-157.5	F	1.925	2743.0
		F	2.627	2801.7
		F	1.830	4021.9
		F	2.287	3533.0
		M	1.808	3628.0

thidin-*p*-nitrobenzoate was soluble in hot alcohol but settled out upon cooling. It was thus impossible to determine its lethal dose by the intravenous route.

Groups of 10 cats were employed for strophanthidin dichloroacetate and -diethylaminoacetate, and groups of 5 cats, for each of the remaining products except the *p*-nitrobenzoate. The results of individual readings are shown in table 1. The geometric mean, that is, the antilogarithm of the mean of logarithms of individual doses, was computed for each ester, and is listed in table 2. For the sake of comparison, the published data (7, 8) on strophanthidin-3-acetate, -propionate, -*n*-butyrate, and -benzoate are also included. From our early work (9, 7), the mean (geometric) lethal dose of the combined data with

strophanthidin in 21 cats is  $325.0 \pm 27.3$   $\mu\text{g}$  per kg. It thus becomes obvious that in cats strophanthidin-3-acetate, -diethylaminoacetate, propionate and *n*-caproate are more potent than the aglycone, strophanthidin. There is no significant difference between strophanthidin-3-dichloroacetate and -*iso*-caproate on the one hand and strophanthidin on the other. In general, when the molecule of strophanthidin is enlarged by more than 6 C-atoms, there is a definite trend for the ester to become less active than strophanthidin. This is very evident in the last six compounds listed in table 2.

In a few preliminary experiments, these esters have been tested in a group of dogs by mouth. While the results are irregular there is a definite indication that strophanthidin-3-acetate, -chloroacetate, -dichloroacetate, -diethylaminoace-

TABLE 2  
*Comparison of mean lethal doses of esters in cats*

STROPHANTHIDIN 3-	MEAN (GEOMETRIC) LETHAL DOSE $\pm$ STANDARD ERROR
	$\mu\text{g}$ per kg
acetate	$183.6 \pm 24.6$
chloroacetate	$1052.0 \pm 104.8$
dichloroacetate	$312.7 \pm 47.7$
diethylaminoacetate	$212.5 \pm 28.9$
propionate	$257.3 \pm 35.6$
$\alpha$ -chloropropionate	$612.0 \pm 135.2$
<i>n</i> -butyrate	$428.3 \pm 31.9$
<i>iso</i> -butyrate	$534.4 \pm 118.7$
<i>n</i> -caproate	$215.5 \pm 24.7$
<i>iso</i> -caproate	$311.8 \pm 43.0$
nicotinate	$968.8 \pm 116.7$
benzoate	$2717.0 \pm 261.0$
<i>p</i> -nitrobenzoate	indeterminable
pelargonate	$423.8 \pm 37.6$
laurate	$634.8 \pm 124.0$
myristate	$983.2 \pm 138.0$
palmitate	$3309.0 \pm 249.0$

A correction of the standard errors of the propionate and *n*-butyrate has been made.

tate, - $\alpha$ -chloropropionate, *n* butyrate, *iso*-butyrate, and *n*-caproate, in the amount of one mean lethal dose determined intravenously in cats, are absorbed by the gastrointestinal tract, as evidenced by nausea, vomiting, and frequently slowing of the heart rate. This also holds true for strophanthidin itself. Further work is justified in order to determine whether or not these esters, and others not studied here, may have clinical application.

#### SUMMARY

Strophanthidin-3-acetate, -diethylaminoacetate, propionate, and *n*-caproate are more powerful than the parent aglycone strophanthidin, as shown in cats. By slow intravenous injection, strophanthidin-dichloroacetate and *iso*-caproate appear to be equally as active as strophanthidin. Esters with acids beyond 6

C-atoms, in the series studied, have a lower activity than strophanthidin. A few members of the series with lower molecular weights are effectively absorbed in dogs following oral administration.

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## THE ROLE OF BLOOD DILUTION IN THE ASPIRIN ANTIPYRISIS

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The mechanisms involved in temperature regulation in the body and in the action of antipyretics in lowering high body temperature have been studied extensively by Barbour and his associates (1-23). In maintaining a normal temperature a balance between heat production and heat elimination is present. In hypothermia or hyperthermia the balance is disturbed. The work of the Barbour school is mainly on the mechanisms of heat elimination and a short account of this is given.

The basic factor is a shift of water from the body surface to the interior or from the interior to the surface. Following external application of cold water shifts from the surface of the body to the interior, as shown in the examination of the brain and liver. The amount of circulating blood is less and becomes more concentrated as shown by greater concentration of red blood cells, hemoglobin and proteins and higher specific gravity of the serum. Because of this and probably an increase of sugar and muscle metabolites, such as lactic acid the osmotic pressure increases. On application of heat the reverse picture is seen. The blood becomes more dilute and the osmotic pressure is decreased. With changes in osmotic pressure are corresponding changes in vapor pressure, a lower osmotic pressure causing a greater vapor pressure and an increase in sweating, resulting in an increase in heat elimination. In the case of exercise and in increased metabolism such as in thyroid disease the increased heat formation is balanced by increased heat elimination. In case of infection such as typhoid and malaria, the heat elimination fails to keep pace with the heat formation.

In the experimental fevers, produced by cocaine, betatetranaphyl amine hay infusion and other pyrogenic agents, Barbour has shown that the water shift from the surface to the interior corresponds to that following external heat application.

The mechanisms involved in heat elimination are controlled by the central nervous system particularly the hypothalamus. Barbour has shown that cooling this area brings about anhydremia and liver hydration heating the opposite effect. As shown in monkeys, these changes are absent or much lessened after lesions in the anterolateral hypothalamus (24). Especially striking is the absence of sweating. No sweat was obtained in these animals either under a rise in body temperature produced by exposure to the hot room at 40 C or during febrile plateaus. The other feature in these operated animals was the persistence of higher osmotic pressure in the plasma, demonstrated by greater figures in the plasma specific gravity and plasma chlorides. In these monkeys with anterior and antero-lateral hypothalamic lesions although during the fever

plateau and during the natural decline of fever a lessening of the blood concentration occurred in the responses to hot environment, yet during those circumstances the values of the plasma specific gravity, never fell to figures in which the blood dilution was adequate to elicit sweat secretion

On giving aspirin or other antipyretics to febrile dogs and rabbits there comes a water shift from the interior to the surface. As is the case where external heat is applied, externally or to the hypothalamus, with the shift there occurs a blood dilution, a lower osmotic pressure and increase in vapor pressure. In the primates a marked increase in sweat secretion is found, the diluted blood acting as a stimulus for this. With lesions in the anterior and antero-lateral hypothalamus, the blood osmotic pressure remained high and the sweat response was absent. The conclusion reached is that the antipyresis from aspirin and related drugs is due to action on the hypothalamus bringing about hydremia, accompanied by sweating and vasodilation.

The experiments reported here were planned to bring together—more clearly the factors involved in aspirin antipyresis, especially the sweating. For this purpose, B-methylcholin which causes sweating through its action on the peripheral sweat nerves, was used as the antipyretic agent and its effect compared to those from aspirin.

**PROCEDURES** Three monkeys (*Macaca Mullata*) ranging in weight from 3.2 to 4.0 kgm were employed. The experimental procedures were as follows:

**Group A**—Monkeys in prone position on padded board at room temperature of  $23^{\circ}\text{C}$  ( $\pm 2^{\circ}\text{C}$ ). After a control period each received by stomach tube 100 cc of a hypertonic 5% sodium chloride solution and 30 minutes later a subcutaneous injection of 0.003 grams of mecholyl per kgm of body weight.

**Group B**—Monkeys tied in prone position and at the same room temperature of  $23^{\circ}\text{C}$  ( $\pm 2^{\circ}\text{C}$ ). After the control period each received by stomach tube 100 cc of distilled water, 30 minutes later a subcutaneous injection of 0.003 grams of mecholyl per kgm of body weight.

**Group C**—Monkeys tied down in the same position and at the same room temperature as Group A and B, received after a control period—only the subcutaneous injection of 0.003 grams of mecholyl per kgm of body weight.

Rectal temperature, respiratory rate, sweat secretion in the same area of the left hand, were recorded at ten minute periods. In a previous paper a procedure has been described (24) which permits the accurate measurement of the sensible and insensible perspiration. In the present series of experiments, however, after the mecholyl injection, sweat secretion occurred so rapidly and in such large amounts, that the capacity of the device for sweat collection was inadequate and only after 40 minutes was it possible to obtain an accurate total value of the water eliminated through the sensible and insensible perspiration. To insure more accurate values for water excretion, the figures from the left hand were checked against water collection from the right hand. From the total it was possible to calculate the amount of water secreted every ten minutes and rebuild the shape of the curve of water excretion.

Effects from mecholyl injection not tested in the protocols were pronounced salivation (the amount not swallowed was from 30 to 60 cc of a thick saliva), lacrimation and diarrhea, urination occurred in some instances. Though all the animals were trained to keep quiet under the experimental conditions, after the injection they all showed a characteristic appearance of fatigue. The symptoms accompanying mecholyl administration declined about 30 minutes after its injection.

Six preliminary experiments were performed to give practise in the procedures followed in carrying out the work reported here. In the 3 sets of experiments described using the three monkeys for each of these the procedure was according to the following Latin Square

1 A	2 A	3 A
2 B	2 B	1 B
3 C	1 C	2 C

As a technique for plasma specific gravity determinations the falling drop method of Barbour and Hamilton was employed (25). For the plasma chloride estimation the Van Slyke and Sendroy method was used (26).

**RESULTS. Temperature** The gradual fall in temperature in the monkeys tied down under the conditions already described was similar to that observed in previous experiments under the same experimental conditions. During the control period (the third half hour that the animal was tied down) the three groups of observations showed a slight decrease in the rectal temperature of 0.1 C. In the group of animals receiving hypertonic salt solution by stomach tube group A the rate of fall in rectal temperature was 0.25°C each ten minute period after the solution was given. Group B of animals receiving distilled water by stomach tube also presented a fall in body temperature of 0.25°C every ten minute period. The subcutaneous injection of mecholyl was followed by a striking fall in temperature in all the cases.

**Respiration** These trained monkeys had a steady respiratory rate previous to the administration of mecholyl and no changes were observed during the control periods after the oral administration either of salt solution or of distilled water. A parallel rise in the number of respirations per minute was obtained in all the cases after mecholyl injection. This rise was more marked during the first ten minutes after the injection and almost completely disappeared, regaining the normal rate after 40 minutes. No significant differences can be described among the three groups of animals.

**Sweat.** In former experiments on monkeys tied down at the normal room temperature there was found a gradual decrease in the sensible and insensible perspiration, and during the control time in the 3 groups this was also observed. It was also possible to confirm that animals receiving hypertonic salt solutions tend to lose even less water by sensible and insensible perspiration. The peripheral sympathetic stimulation of the sweat glands produced by mecholyl was apparent three minutes after the administration, reached its peak between 10 and 17 minutes and declined from then on to almost normal values at the end of 40 minutes. It is interesting to point out that in the first experiments due to the low air flow circulating in the device collecting water the curve of secretion showed a gradual rise with a tendency to form a plateau. In the others the actual curve of sweat secretion reached a peak 10 minutes after mecholyl administration falling sharply after that period. The total amount of water excreted by the skin of the monkey's hand varied between 0.600 and 1 gram during 40 minutes. Higher values of water excretion were found when the animals received water before mecholyl injection, and the lower ones were obtained when

TABLE A

Normal monkeys receiving at ▲ 100 cc NaCl sol 5% and at ■ 0.003 gm mecholyl/kg body weight

(Average of 3 observations)

TIME (MINUTES)	10	20	30	▲ 40	50	60	■ 70	80	90	100
Temperature (°C)	38.9	38.9	38.9	38.7	38.6	38.3	38.2	37.9	37.7	37.2
Respirations/m	46	46	46	46	46	46	80	73	61	51
Sweat (H <sub>2</sub> O gms)	0.041	0.036	0.035	0.033	0.029	0.033	0.050	0.236	0.282	0.205
Plasma specific gravity		1.027				1.0278				1.0291
Plasma chlorides (mEq/l)		102.5				108.8				112.0

TABLE B

Normal monkeys receiving at ● 100 cc of water and at ■ 0.003 gm mecholyl/kg body weight

(Average of 3 observations)

TIME (MINUTES)	10	20	30	● 40	50	60	■ 70	80	90	100
Temperature (°C)	38.9	38.8	38.8	38.7	38.6	38.5	38.4	38.1	37.8	37.3
Respirations/m	35	35	35	35	35	35	66	47	45	40
Sweat (H <sub>2</sub> O gms)	0.037	0.039	0.04	0.043	0.043	0.048	0.076	0.374	0.318	0.216
Plasma specific gravity		1.028				1.0273			1.283	
Plasma chlorides (mEq/l)		106.9				100.5			104.0	

TABLE C

Normal monkeys receiving at ■ 0.003 gm mecholyl/kg body weight

(Average of 3 observations)

TIME (MINUTES)	10	20	30	40	50	60	■ 70	80	90	100
Temperature (°C)	38.8	38.8	38.8	38.8	38.7	38.7	38.6	38.4	37.7	37.3
Respirations/m	35	35	35	35	35	35	60	53	41	37
Sweat (H <sub>2</sub> O gms)	0.037	0.031	0.026	0.036	0.028	0.027	0.068	0.354	0.280	0.193
Plasma specific gravity					1.0277		1.028			1.0308
Plasma chlorides (mEq/l)					106.0		103.2			103.1

the same animals received previously sodium chloride hypertonic solution (5%)  
The corresponding values for each group of animals appear in tables A, B, and C

*Plasma Specific Gravity* Considerable importance was given during this work to the study of the plasma specific gravity values. The monkeys receiving hypertonic salt solution showed a rise in the plasma specific gravity when compared with that presented by the same animals during the control period. A greater rise was present after mecholyl injection when the blood samples were taken at the end of the sweat production. The monkeys receiving distilled water presented slightly lower specific gravity values than those obtained in the same animals during the previous control period but after the mecholyl injection these monkeys also gave higher plasma specific gravity figures. Group C of control animals, which received neither water nor oral hypertonic salt solution, after injection of mecholyl also responded with strikingly high values in the blood plasma specific gravity. All the animals therefore whether they presented blood dilution provoked by water administration or blood concentration due to hypertonic salt solution exhibited anhydremia following the great sweat production under sympathetic stimulation.

*Plasma Chlorides* In the monkeys of Group A which received salt higher plasma chlorides were present after the administration of the hypertonic solution than before the salt was given. A further increase in blood chlorides was observed after mecholyl injection. In the Group B the plasma chlorides decreased after the water was given but after the subcutaneous injection of mecholyl the plasma chlorides tended to return to higher figures. The animals of the control group C which received neither water nor salt exhibited a very slight fall in plasma chlorides after mecholyl injection.

*DISCUSSION* The observation made in this experiment that under sympathomimetic stimulation a loss of water by sweating produces a fall in temperature confirms the important role of this mechanism in the heat loss of the antipyresis. The reverse effect namely a decrease of the perspiration and an increase of temperature after atropine has been recently demonstrated (27). Apparently this mechanism of heat loss (peripheral vasodilation and an increase in insensible and sensible perspiration) is similar to that elicited by antipyretics like aspirin, for with aspirin antipyresis in febrile monkeys there is also observed vasodilation and a steady increase in sweating. However, an important difference is clearly present between the sweating produced by sympathomimetic drug like mecholyl and an antipyretic like aspirin. The water shifts accompanying the temperature fall following mecholyl are always indicated in the blood by a steady rise in the blood concentration, produced passively by the water lost during the increase in perspiration. On the contrary during aspirin antipyresis a blood dilution and lower values of the blood osmotic pressure were demonstrated. It must be mentioned that the mechanism of aspirin antipyresis is simply an elicitation of the already present bodily responses either to fever or to a hot environment. On the other hand the fall in temperature obtained by sympathetic stimulation of the sweat glands is a passive phenomenon. The heat loss by water evaporation is followed by a blood concentration resulting in higher values for the blood osmotic pressure. But during the aspirin antipyresis active water shifts occur drafting water mainly from the liver and the muscle cells into the peripheral

blood These water movements present in the monkeys can also be expected in human subjects

Monkeys suffering lesions in the anterior and antero-lateral hypothalamic areas are unable to respond with defense mechanisms either to a hot environment, fever or aspirin administration Even more, these animals are unable to produce sweat under these circumstances The explanation of this lack of response of the heat dissipation mechanisms to the antipyretics is found in the changes occurring in the plasma specific gravity These primates, after suffering the hypothalamic lesions, suddenly show a high increase in the blood specific gravity and are unable to lower it under the stimulation produced by fever, heating or aspirin administration (24) Under these levels of homeostasis the high osmotic pressure opposes the loss of water from the blood and little or no sensible and insensible perspiration is produced Therefore, the presence of a high osmotic pressure in the plasma will prevent the loss of water As the hypothalamic control of blood osmotic pressure is very sensitive to slight changes in blood specific gravity, water administration tends to increase perspiration, while the ingestion of hypertonic salt solution tends to reduce the sweat secretion These findings provide a logical basis for the treatment of fever by oral administration of large amounts of water (28)

An understanding of the quite different mechanisms of action of mecholyl and of aspirin permits the reconstruction of the sequence of events in the antipyretic action of the latter Its action has its focal point in the anterior hypothalamic areas The threshold responsiveness of these to temperature changes and to changes in blood concentration is altered, and there occurs a shifting of water from the tissue cells to the peripheral and skin circulation With the blood dilution comes a lowering of osmotic pressure and a rise of vapor pressure bringing about a greater elimination of water whose evaporation from the surface removes heat from the body Accompanying the blood dilution is a marked increase in the activity of the sweat glands, an especially important factor in the water elimination and heat loss in aspirin antipyresis

#### SUMMARY

The anterior hypothalamic areas, by producing blood dilution and water shifts, indicate the body responses to environmental heating, fever and aspirin administration An opposite effect, i e., plasma concentration is present as a response to cold and during the onset of fever

Mecholyl by means of water losses through sweating and vasodilation, produces a fall in temperature Its action is enhanced by previous water administration and reduced by hypertonic salt solutions

Accompanying the marked sweat secretion produced after mecholyl injection a rise in the respiratory rate was observed

The stimulation of this heat loss mechanism is not accompanied, as in aspirin antipyresis, by plasma dilution, but a plasma concentration occurs

After demonstration of these differences of action between this parasympathomimetic drug and the true antipyretics, the mechanism of aspirin antipyresis

can be described in the following manner. During the febrile condition, through a hypothalamic stimulation starting in the anterior nuclei, water is shifted from the interior cells to the periphery and a plasma dilution occurs. This activates heat loss mechanisms such as increased perspiration. Aspirin acting upon the anterior hypothalamic nuclei enhances the process increasing further the plasma dilution and, therefore increasing the loss of water through the sensible and insensible perspiration. Aspirin through this central action alters the threshold responsiveness of the hypothalamic nuclei toward lowering body temperature by heat loss. It is considered that the lowered osmotic pressure which accompanies blood dilution is an important factor in the hypothalamic function.

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# OBSERVATIONS ON THE ANALGESIC EFFECT OF MORPHINE DURING CONTINUED DAILY ADMINISTRATION OF SMALL AND UNIFORM DOSES TO DOGS<sup>1</sup>

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Results of experimental studies in animals indicate that tolerance to various effects of morphine develop slowly if at all when morphine is administered in small and uniform daily doses (6). Few investigators, however, have studied changes in the pain threshold raising effect of morphine under such conditions. Further information on this problem appeared desirable in a search for means by which to improve the clinical management of chronic or recurrent pain. The present study was undertaken with such a purpose in mind.

**REVIEW OF THE LITERATURE** Möhrke (5) reported that injections of 70 to 100 mg of morphine on alternate days for three weeks produced a decrease in the analgesic power of the morphine in human subjects. The decreased analgesia measured by electrical stimulation of the finger was considered to represent a developing tolerance to the drug.

Hoefer (4) and Grünthal and Hoefer (5), using the Von Frey method, observed in six normal human subjects that morphine injected daily in doses of 10 to 20 mg lost its analgesic properties within eight to fourteen days. The authors noted that the pre-administration levels of pain threshold tended to rise during the period of daily injections. The analgesic effect and the pre-administration levels returned to their original values within ten days after withdrawal.

Eddy and Himmelsbach (1) found that in cats the analgesic effect of 20 mg of morphine sulfate per kilogram body weight did not diminish during two months of daily injections. The exciting and emetic effects disappeared during the first month of injections. The pain thresholds, however, were measured in terms of the amount of pressure which had to be applied to the cat's tail to produce a reaction. Aside from species differences, the discrepancy between the results and clinical experience may arise from the fact that such a method of producing pain necessarily also produces sensations of pressure. It has been reported by Hoefer (4) that the effect of morphine upon the sensation of pressure is not changed by prolonged administration.

**METHODS** *The general design of the experiment* This study was conducted on eight dogs. Control determinations of the tooth pain threshold were made at intervals during a period of sixteen weeks before and after the fifty day period of morphine administration. During the period of morphine administration, each dog was given subcutaneously 30 mg of morphine sulfate per kilogram body weight at the same time every evening. The pain threshold was deter-

<sup>1</sup> This work was aided in part by a grant from Smith, Kline & French Laboratories

mined twice weekly just before the injection of morphine and at thirty, sixty and ninety minute intervals after the injection and at the same time (7 00 p.m.) of the day and under the same conditions under which the control determinations were made. After the conclusion of the fifty day period of morphine administration, the elevation of the pain threshold after morphine was determined on the thirtieth and fortieth days.

The animals were fed during the morning. All determinations were made in a closed room with only the operators present the only known uncontrollable factors being the barometric pressure and the humidity. The presence of all conditioning cues were avoided and carefully looked for. The animals were trained to stand in a stock before pain threshold determinations were started and each animal was placed in the same relation to the others in the group during each test, other details need not be mentioned.

*The algometric method used* The algometric method used has been published in detail elsewhere (2) along with an analysis of various algometric methods. It consisted of applying an uninterrupted alternating current in peaks of variable but controlled voltage through metal fillings in the cuspid teeth of the dogs. There were two fillings in each tooth and in the test two pincher like platinum electrodes were applied to the fillings in one and the same tooth. The path of the current was probably directly through the tooth, since when the current was applied to metal fillings in our teeth or those of other human subjects the only sensation perceived was projected to the tooth stimulated.

*The choice of the criterion of pain.* When such a current is applied to a metal filling in the tooth of a human subject and then gradually increased, the first sensation is difficult to describe it simulates a prick which is not painful. A further slight increase in current causes a pain sensation. It is believed that the pulps of teeth are innervated only by pain nerves. This is our opinion based on the repeated stimulation of the pulp through fillings in more than fifty human subjects.

In man the sensation of pain is experienced with much less current than that which causes an avoiding reflex. Thus it is reasonable to assume as must be done in any pain study on animals, that a dog senses pain at or below the level of the threshold at which an avoiding reflex is elicited. Since according to existing evidence, the pulp is innervated only by pain nerves an avoiding reflex, an objective phenomenon produced by stimulating a tooth should be more reliable for the determination of pain threshold than the subjective report of a human subject. This should obtain particularly when drugs are used which effect the subjective feeling tone or the mental attitude.

Dogs vary in regard to the type of avoiding reflex they manifest when the tooth pulp is stimulated. Some dogs contract the corner of the mouth some contract the tongue and others move the mandible. The first noticeable contraction standard for the dog was interpreted as indicating that the pain threshold had been reached. Three threshold readings were made at each interval determination, the lowest peak voltage which produced the standard response three times in succession was accepted as the measure of the pain threshold for

any interval determination. The threshold of each of two cuspid teeth was recorded and the average of the two was considered to be the threshold.

*The nature of the control.* When the threshold was determined in the control period and in the morphine period, the initial threshold was determined about fifteen minutes after the animals were placed in the stock and again at thirty, sixty and ninety minutes. In the morphine period, the morphine was injected after the initial threshold had been determined. Determinations were not made for a longer period than ninety minutes to avoid the effects of fatigue in the dogs.

*The control data.* The method we employed yields three types of control data, namely, the initial threshold value for each test, the threshold values (zero, thirty, sixty, ninety minutes) for a blank test in which no drug was given, and both groups of control data for sixteen weeks before and sixteen weeks after the fifty-day period of daily morphine administration.

TABLE 1

*Showing the mean, the average deviation, and maximum single deviation from the mean of twenty-eight initial control tests, made over a period of sixteen weeks, in volts*

DOG	AVERAGE	AVERAGE DEVIATION	MAXIMUM SINGLE DEVIATION
1B	1 21	0 19	0 41
2C	0 66	0 12	0 21
3F	1 08	0 06	0 17
4L	1 16	0 15	0 34
5Q	1 69	0 17	0 41
6S	0 61	0 08	0 21
7T	1 47	0 17	0 46
8W	0 87	0 21	0 33
Average	1 09	0 14	

The *initial threshold value* represents the first value determined fifteen minutes after the trained animals were placed in their stocks. It provides information regarding the extent to which the pain threshold of the teeth varies in each dog from test to test, the tests being performed on Tuesday and Friday. The initial mean threshold value of each dog is shown in table 1 with the average deviation from the mean, the maximum single variation is also shown in the table. The initial test threshold from test to test may vary 0.2 volt above or below the mean, but only occasionally more. The cause of this is uncertain, but the results shown in the table (table 1) are representative of the variations we have seen in eleven other dogs we have used for other algometric studies. The *initial threshold value* for each dog also serves as a control value with which to compare the analgesic action of the drug to be administered.

The *blank control test* values consist of the algometric readings made initially and at thirty, sixty and ninety minutes when no drug is given. These data provide information regarding the effect, if any, of standing or sitting in the stock, or the extent to which the threshold varies over a period of ninety minutes.

The four values may show a spread in the dog of 0.1 volt, but rarely more. The average of a series of blank control test values for this group of eight dogs is shown in figure 1 in which it is contrasted with the average of the test values obtained after the injection of 3 mg of morphine sulphate per kilogram body weight. It is to be noted that on the average, without drugs, the threshold tends to decline with time during the test period.

**RESULTS** *The analgesic effect of morphine sulphate injected at three to ten day intervals* Before starting the daily injections of morphine it was considered advisable to ascertain the analgesia caused by the dose of morphine to be injected daily (3 mg per kilogram). This dose of morphine was given subcutaneously to the dogs on October 29 November 9, 12 and 16 the latter date representing the first day of the fifty-day period of daily injections of the same dose. It was assumed that three to ten-day intervals were sufficient for the effect of morphine

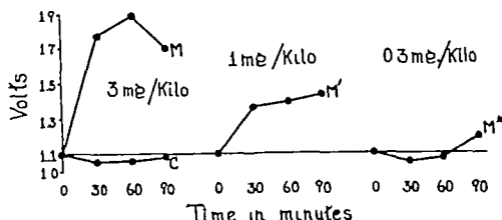


FIG 1 SHOWING AVERAGE RISE IN THRESHOLD OF THE 8 DOGS AFTER RECEIVING THEIR FIRST DOSE OF MORPHINE 3 MG PER KILO BODY WEIGHT, THE EFFECTS OF OTHER DOSES AND THE AVERAGE OF CONTROL DOSES

'C' is the curve of the average control data from 43 tests on the 8 dogs. 'M' shows the average rise in pain threshold of the 8 dogs after the single dose of morphine. 'M' and 'M'' show effects of smaller doses.

on pain threshold to disappear. Four weeks (February 4) and about six weeks (February 15) after the end of the fifty-day morphine period, the analgesic effect of the dose of morphine was again determined. It was believed that six morphine control injections, four before and two after the fifty-day period, would be adequate, since it is well known that 3 mg per kilogram of morphine has an analgesic action on the dog.

The algometric data for the tests on November 12 and 16 and February 4 and 15 for the eight dogs are shown in figures 2 and 3 in the volts required to produce the standard threshold avoiding reaction in each dog. The extent of analgesia varied in the different dogs and in the same dog but its peak was usually observed thirty minutes after the injection.

In figure 4 the same data are illustrated by showing the average analgesia recorded over the ninety minute period. For example, using the curve in figure 2 on Dog 4L on November 12 at thirty minutes the threshold was increased 1.35 volts at sixty minutes 0.75 volt at ninety minutes, 0.65 volt, the sum is

275 volts, or an average of 0.9 volt, as represented by the column for November 12, Dog 4L in figure 4

*The analgesic effect of consecutive daily injections of the same dose of morphine sulphate* The first algometric test after daily injections had started was made on November 19 or on the fourth daily injection. Others were made as indicated in figures 2, 3 and 4

Some tolerance or decreased analgesic effect of morphine was observed in four of the eight dogs on the fourth daily injection (7I, 1B, 6S, 2C), by the eighth

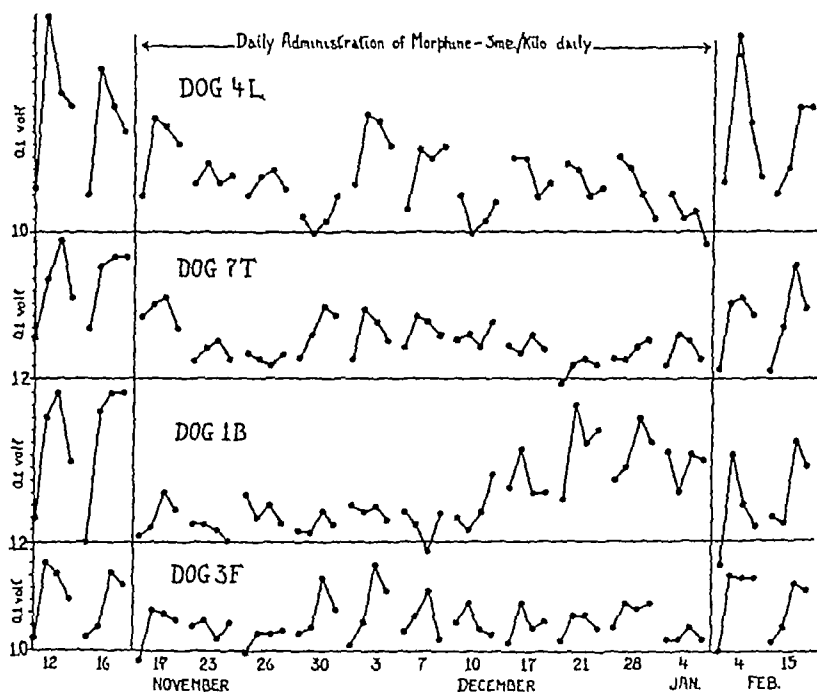


FIG 2 SHOWING THE ALGESIMETRIC DATA ON MORPHINE ADMINISTRATION BEFORE, DURING, AND AFTER THE 50 DAY PERIOD OF DAILY MORPHINE ADMINISTRATION

The first value in each curve is the initial threshold value for the test before the morphine was injected

day it was decisively present in six of the eight dogs (4L, 7I, 1B, 3F, 6S, 2C). In dogs 5Q and 8W it was not decisively present until the twenty-fourth and thirtieth days, respectively

The decreased analgesic effect of the morphine is not correlated in the individual animals with a high initial test threshold, that is, the control value obtained before injecting the morphine. This is shown by inspecting the records of Dogs 8W, 5Q and 1B particularly. However, when the initial thresholds of all dogs are averaged for the pre- and post-morphine period and compared with those of the morphine period, it is found that the averages of the initial thresh-

holds during the morphine period are significantly higher than those of the pre- and post-morphine periods. For example figure 5 shows the average initial threshold value for the dogs for each test from July 8 1943 to April 28 1944. The mean initial threshold for all the tests during the morphine period is 1.39 volts, for the control periods 1.117. The difference is 0.273 with a probable error of  $\pm 0.02$  which is definitely significant. Also when one compares the initial threshold of each dog during the morphine period in figures 2 and 3 with the control period averages for each dog in table 1 it is found that the initial

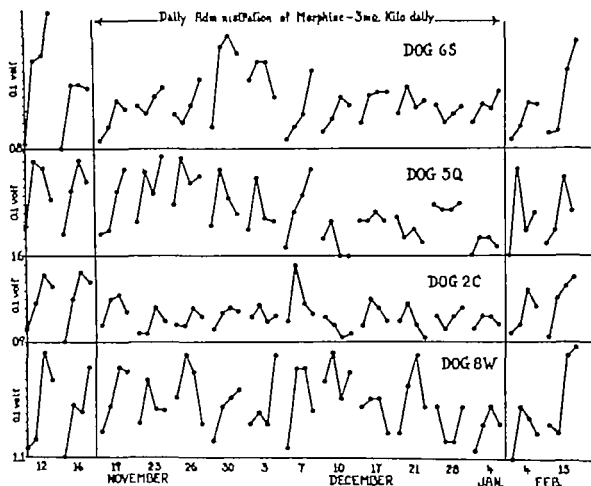


FIG 5 SHOWING THE ALGESIMETRIC DATA ON MORPHINE ADMINISTRATION BEFORE, DURING AND AFTER THE 50-DAY PERIOD OF DAILY MORPHINE ADMINISTRATION

The first value in each curve is the initial threshold value for the test before the morphine was injected

threshold of every dog during the morphine period is above that of the control period. To what this may be due is uncertain. We believe that it is due to some effect of the morphine though we have no control to determine whether it is a seasonal change. It is because of this phenomenon that the data are presented as they are in figures 2, 3 and 4 since when so presented they show that a high initial threshold does not limit the extent of the analgesic response. This is so obvious from an inspection of the individual data that statistical treatment is unnecessary.

A study of the curves in figures 2 and 3 and especially the columns in figure 4

shows that in some tests the injection of morphine apparently decreased the pain threshold. It has been shown, figure 1, that the threshold tends, on the average, to decrease when no drug is given, and it has been stated that the variation in successive determinations in a test without a drug is  $\pm 0.1$  volt. Hence, all the decrements in threshold shown in figure 4, with the exception perhaps of Dog 4L, can be interpreted as due to a lack of analgesic action of the

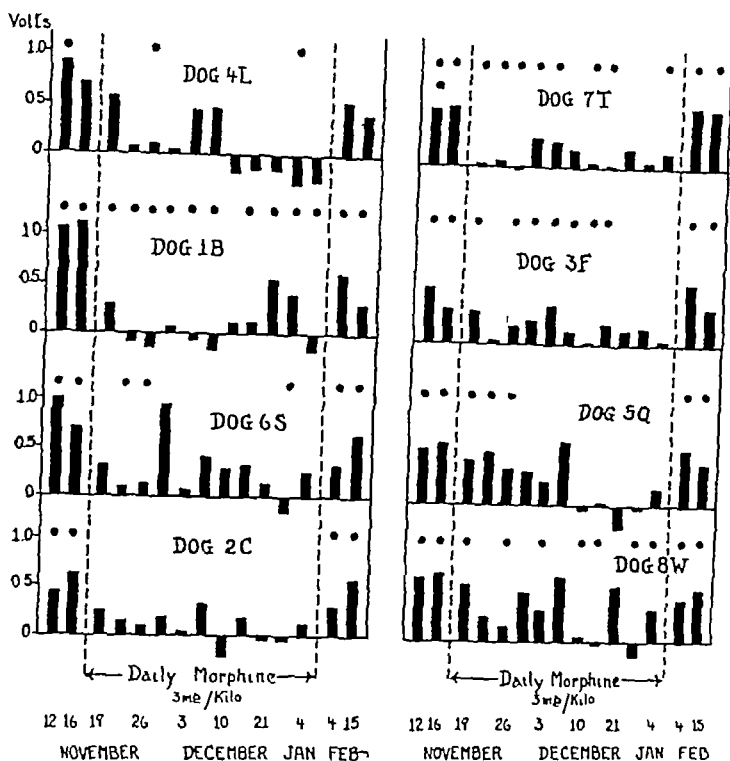


FIG 4 SHOWING THE AVERAGE EXTENT OF ANALGESIA OVER THE 90-MINUTE PERIOD AFTER INJECTION, USING THE INITIAL CONTROL THRESHOLD OF EACH TEST AS THE BASE OF ESTIMATING THE AVERAGE ANALGESIA  
Black circle indicates occurrence of vomiting

morphine. This is not applicable to some of the tests (e.g., 4L, figure 2, December 10, 17, 21, 28).

*Periodicity in tolerance* is evident when figures 2, 3 and 4 are inspected. This is quite evident in all the dogs except 5Q and 8W, in which tolerance did not develop until late in the period, though we believe it is evident in Dog 8W. It probably would have become manifest in 5Q if the injections had been continued. In Dogs 4L, 1B, 3F, 6S and 2C the analgesic response to morphine changes from no response to a response clearly within the normal range.

*General observations* At the start of the daily morphine injections, the average weight of the dogs was 11.6 kg. During the period the average loss of weight was 0.95 kg. The dose of morphine, however, was kept at 3 mg per kilogram of initial body weight. By the thirtieth day after withdrawal of the drug, all animals had regained their initial weight.

Records of vomiting were kept. The tests in which vomiting occurred are recorded in figure 4. All dogs vomited on October 29 and 12, seven on the 16th. Dog L was the only one that did not vomit when injected one month after withdrawal on February 4 and February 15. Tolerance to the emetic action of morphine was complete in two of the eight dogs, dogs 2C and 5Q, and was

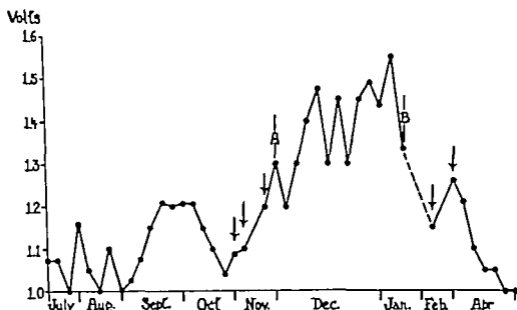


FIG. 5 SHOWING THE AVERAGE INITIAL TEST CONTROL THRESHOLD VALUES FOR EACH TEST OVER A 16-WEEK PERIOD BEFORE AND 16-WEEK PERIOD AFTER THE PERIOD OF DAILY MORPHINE ADMINISTRATION

The controls started July 8. Four morphine controls were started October 29 (note arrow) at not less than 4-day intervals, the last being performed on November 16 on which day (A) the daily injections started. The daily injections continued to January 4 (B). The injection of morphine was repeated on February 4 and again on February 15. Blank control tests without any drugs were started on April 5 and continued as indicated to April 28.

practically absent in two, dogs 1B and 8W. In the other dogs vomiting was intermittent. The daily records of vomiting show that on the twenty-ninth, twenty-seventh and thirty-seventh days no dogs vomited; on the forty-eighth day all vomited and on the forty-ninth only one. No attempt was made to ascertain the role of conditioning in vomiting, but one dog was definitely conditioned since it vomited occasionally before the injection on the sight of the syringe.

Defecation followed the initial injections in five of the eight animals. Thereafter it occurred rarely but occurred again in the five animals on the injection made thirty days after withdrawal (February 4 and 15).

A conditioned salivary reflex developed in all animals and persisted through

out the period of daily injections. It also appeared on the first day after withdrawal, when saline solution was injected in place of the morphine. At first the dogs salivated only after the morphine injections, but shortly started to salivate before the injections. The post-injection salivation then decreased so that at times the dogs salivated more before than after the injection. The post-injection salivation, though not measured, appeared to decrease and increase or to vary from day to day during the period of daily injections.

A marked depression was apparent in all animals following the first few injections of morphine. They could be aroused but quickly returned to a relaxed and semi-comatose condition. The intensity of the depressant effect decreased after several daily injections and thereafter appeared to increase and decrease periodically. The effect was not entirely absent at any time. There is no satisfactory method of measuring the degree of depression.

On several occasions, restlessness was observed in some of the animals just before the injection of morphine. The restlessness was followed by depression after the injection. When the daily injections were stopped after fifty days, withdrawal symptoms were negligible. For three days after the morphine was stopped the dogs received injections of normal saline at the accustomed time. The dogs showed some restlessness before these injections. The restlessness did not diminish after the saline injections. No salivation followed these injections.

**DISCUSSION** This investigation was primarily undertaken to ascertain whether tolerance to the analgesic action of morphine could be demonstrated in the dog. We thought the observations made by Eddy and Himmelsbach (1) on the cat were not sufficiently discriminative in the light of the observations of Hoefer (4) in man to warrant the belief that man develops tolerance to the analgesic effect of morphine and the cat does not. We believed that the method of eliciting an avoiding reflex by stimulating the nerves of a tooth, which has only pain nerve-endings, is more discriminative (it was in our experience) than eliciting avoiding reflexes by applying pressure to the tail, which contains both pain and pressure nerve-endings.

We believe our data unequivocally demonstrate that the dog, in so far as tooth pain is concerned, manifests tolerance to the analgesic effect of morphine.

Our data, however, unexpectedly showed that the time of onset and the extent and duration of the tolerance to the analgesic effect of morphine is subject to variation. The tolerance became complete and then partially or completely disappeared in four days, though the injections of morphine at the same dosage level were continued. An explanation of this phenomenon is not available, since an adequate explanation for tolerance to any effect of morphine has not been established.

The only investigators who have injected dogs with 3 mg of morphine sulfate per kilogram body weight daily over a long period are Tatum, Seevers and Collins (6). We have confirmed their observation that complete tolerance to depression and drowsiness does not occur under such a condition. These investigators do not report whether the degree of depression and drowsiness varied with injections from time to time. Our impression is that it does, the same is

true of the extent of the post-injection salivation. In order to explain the discrepancies in the reports on the development of tolerance to the excitatory effect of morphine in the cat, Krueger Eddy and Sumwalt suggest that 'tolerance to the exciting effect can develop if only small doses are employed. It is possible that at the dosage level we employed in dogs we may be dealing with what may be termed the threshold of the tolerance mechanism at which level the extent and duration of the excitatory effects and of the tolerance to the depressive effects of morphine may vary and hence influence the extent of the depression and analgesia. However we find little evidence to support the concept that the animals became tolerant to the excitatory effects of morphine on that basis it is impossible to explain the decrease in pain threshold after the injection of morphine in Dog 4L and the failure to observe analgesia in numerous instances. We suspect that the variations in response are due to asynchronous variations in the extent and duration of the excitatory effects and of the tolerance to the depressive effects of morphine.

#### SUMMARY

Eight dogs were each given 30 mg of morphine sulfate per kilogram body weight subcutaneously daily for fifty days. General reactions were observed and changes in tooth pain thresholds were determined by electrical stimulation. The tooth pain thresholds were determined for twelve weeks before and after the period of morphine administration.

Reactions such as vomiting salivation and defecation were reduced after the first few injections and thereafter varied irregularly.

Elevation of pain threshold by the morphine was at first marked, becoming less on successive injection and then again greater, the effect tending to vary periodically. The pre-administration pain threshold tended to be higher for the group during the period of daily administration of morphine.

It is concluded that continued daily administration of a uniform dose of morphine produces tolerance to the analgesic effect of morphine in the dog but that the tolerance is not lasting, disappearing and reappearing periodically as daily administration is continued.

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# OBSERVATIONS BEARING ON THE MECHANISM OF THE ELIMINATION OF QUININE AND ATABRINE FROM THE CIRCULATION AND TISSUES<sup>1</sup>

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It has been observed in man, dog and cat that 90 per cent of quinine injected intravenously leaves the blood within 5 minutes (1, 2, 3). Hatcher and Weiss believed quinine is quickly and temporarily fixed by the capillaries (4). In the case of atabrine, Hecht found it to be taken up by the organs with which it first comes into contact—by the lung in intravenous injection and by the liver in oral administration (5). In tissues, quinine is to a large extent destroyed enzymatically (6, 7, 8), however, it is not understood how atabrine is metabolized in the body. The purpose of the present investigation is to find out the mechanism involved in the initial rapid disappearance of these alkaloids from the circulation or tissues.

The experiments were conducted in chickens after partial blockage of the reticulo-endothelial system, in dogs with hepatic injury, in rabbits with leukopenia, and following evisceration, and in heart lung preparations. These preparations were employed in order to investigate whether the reticulo-endothelial system, the blood elements of the bone marrow or the capillary wall takes part in the initial elimination process. The rate of elimination of quinine, and atabrine from the circulation was determined during the first two hours following intravenous injection. In heart-lung preparations, the amount of alkaloid in the tissues was also determined.

**EXPERIMENTAL** *I Chemical Analyses* A quinine and its fluorescent degradation products were differentially determined by a method which was devised by us for the present investigation. In brief, quinine together with its degradation products was extracted from the blood and tissue samples at pH 8.5 with ethylene dichloride. For quinine alone the blood and tissues were first digested with 2 per cent NaOH, and in this alkaline medium the extraction with ethylene dichloride was carried out. The amounts of these substances were determined fluorometrically with the Coleman photofluorometer (Model 12).

*Quinine plus its degradation products* Into a 30 cc glass stoppered centrifuge tube containing 4.5 cc water and 1 cc M/2 Na<sub>2</sub>HPO<sub>4</sub>, there was delivered 0.5 cc blood, followed by 10 cc ethylene dichloride (purified by shaking 10 minutes with norit). Oxalated blood may be used. In the case of tissues, the fresh material was homogenized with water in a Waring blender. One gram of tissue was made up to a final volume of 10 cc, 2.5 to 5.0 cc of the tissue suspension were taken for each determination. Into the suspension in the centrifuge tube, 1 cc M/2 Na<sub>2</sub>HPO<sub>4</sub> was added and a sufficient amount of water to make a total volume of 6 cc. This was followed by 10 cc ethylene dichloride. For larger quantities of blood and tissues, the reagents were increased proportionally. The contents were

<sup>1</sup> The work described in this paper was done under contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the University of Chicago.

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shaken vigorously for 4 minutes and centrifuged at a moderate speed for 15 minutes. The aqueous layer and the precipitate were drawn off carefully by suction connected to a water pump. In some instances, as with the chicken blood where the precipitate was jelly like and could not be removed easily without carrying along a considerable amount of the ethylenedichloride the precipitate was broken into parts with a glass rod and a portion of it large enough to allow the entrance of a pipette was drawn off by suction.

Six cc of the extract were pipetted into a test-tube cuvette and 2 cc. acid-alcohol (1.92 parts of absolute alcohol to 0.08 parts of 10 N  $H_2SO_4$ ) were added to it. The amounts of quinine and the degradation products were then determined with the Coleman photo-fluorometer using B<sub>1</sub> PC-1 or B<sub>1</sub>S PC-1 filters.

**Quinine alone** The amount of blood or tissue suspension was usually the same as that for the determination of quinine and its degradation products. Enough water and 4% NaOH were added to the centrifuge tube to make a total volume of 6 cc containing 2% NaOH. The contents were heated in a water bath for one hour the tube being covered with a boiling cap in order to prevent an excessive loss of water by evaporation. After cooling 10 cc ethylene dichloride were added followed by the procedure given in the above section. Extraction at this high alkalinity sometimes resulted in the formation of an emulsion at the interface especially when a large quantity of blood or tissue had been used. This however rarely happened with 0.5 cc blood or 2.5 cc. of the 10% tissue suspension. Whenever an emulsion had formed the upper aqueous layer was drawn off and replaced with 5 cc water. The contents were shaken gently for a few seconds so as to break up the emulsion. This manipulation and centrifugation did away with the colloidal condition without resulting in a diminution of the accuracy of the method.

**B Atabrine** was estimated by the single ethylene dichloride extraction procedure of Brodie and Udenfriend (9) with slight modifications to meet our experimental conditions. The extraction was carried out at pH 8.5 with M/2  $Na_2HPO_4$  in a 30 cc glass-stoppered centrifuge tube B<sub>1</sub> PC-9 filters in a Coleman photofluorometer were used for isolating the fluorescence of atabrine in ethylene dichloride acidified with glacial acetic acid.

**II Technical Procedures. A Blockage of the reticulo-endothelial system.** Chickens were injected with 5 cc of 1% norit in physiological saline daily for four days. They were used 24 hours after the last injection.

**B Liver damage** Dogs were given 10 cc  $CCl_4$  per kilogram by stomach tube. At the time of experimentation the animals showed obviously toxic signs of hepatogenic degeneration such as pronounced jaundice a prolonged retention of the injected bromanaphthalein in the circulation and an increased coagulation time of the blood.

**C Production of leukopenia.** Rabbits were exposed to nitro-mustard gas at such a concentration and duration that only the white cells in the circulation were decreased in number (500-1000 per cm<sup>3</sup>).

**D Evisceration.** In rabbits under urethane anesthesia, the entire gastro-intestinal tract spleen liver pancreas adrenals gonads and kidneys were removed. In one experiment the right innominate the left carotid and the left subclavian arteries were also ligated.

**E Heart-lung preparation of the rabbit *in situ*.** The technique is essentially that of Knowlton and Starling (10) for the cat with one modification which is necessitated by the presence of two precaval veins in the rabbit. The external circuit was connected to the preparation from the right carotid to the left precaval vein. It was found essential that cannulation of the left precaval vein be performed prior to the ligation of the right subclavian artery and the right precaval vein, thus providing a better venous return to the right auricle. Instead of adding heparinized blood to the heart-lung preparation 75 cc of heparinized Ringer Locke solution were administered into the prepared rabbit by way of the left precaval vein just before the ligation of the abdominal aorta. The amount of the heparinized saline (about 50 cc) in the external circuit plus the quantity of blood drawn into it from the animal's own body was sufficient to keep the heart-lung preparation functioning physiologically for the period required by the experiment (1-2 hours).

The alkaloid was injected into the preparation through the precaval vein and the blood for analysis was drawn from the carotid artery.

**RESULTS AND DISCUSSION** Photofluorometric determination of quinine and its degradation products The fluorometric readings were standardized against ethylene dichloride extracts of known quinine concentrations, prepared under conditions identical with those for blood and tissue analyses The zero reading

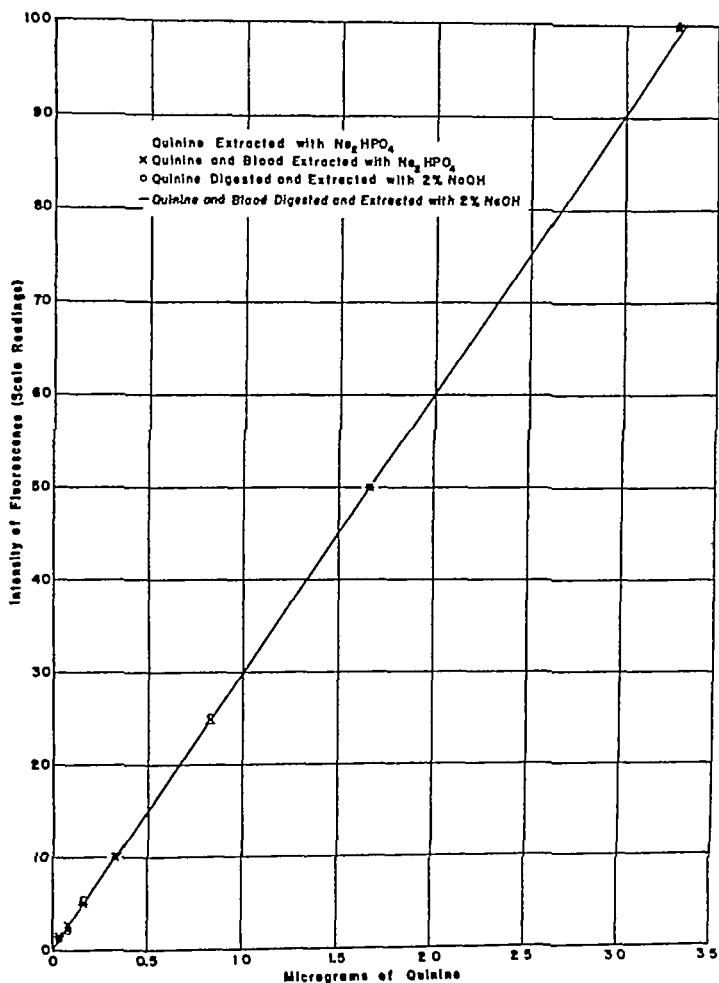


FIG 1

on the instrument was adjusted with a solution containing 6 cc ethylene dichloride and 2 cc acid-alcohol A linear relation was obtained between the intensity of fluorescence of quinine in ethylene dichloride in the presence of  $\text{H}_2\text{SO}_4$  and the amount of quinine taken for extraction (fig 1)

Owing to the unknown nature of the quinine degradation products, the

amounts of these substances were determined with the same filters using quinine as the standard

The recovery of the added amounts of quinine from the blood by both procedures is shown in figure 1 in which the intensity of fluorescence of quinine in the extract was plotted against the amount of quinine taken for extraction with and without 0.5 cc. blood of the dog. It will be seen that the amount of quinine in the blood, as low as 0.2 microgram per cc., may be accurately determined by comparing it with a standard prepared under identical conditions.

Data for the recovery of quinine from tissues of rabbit are given in table 1. The lower values obtained from the lung and the liver suspensions at pH 8.5 might have been due to some loss by enzymatic destruction of quinine in these media.

TABLE 1  
*The determination of quinine in tissues*

TISSUES (0.25 gm.)	QUININE HCl ADDED  <i>micrograms</i>	% RECOVERY (EXTRACTION MEDIA)	
		NaH <sub>2</sub> PO <sub>4</sub>	2% NaOH
Heart	20	101.0	95.9
	20	99.0	94.4
Lung	20	94.4	100.0
	20	94.4	102.0
Liver	20	93.4	101.0
	20	94.0	107.0
Spleen	20		99.8
	20		100.2
Muscle	20	100.1	104.0
	20	98.0	100.8
Average.		96.7 $\pm$ 1.11	100.5 $\pm$ 1.14

\* First digested with 2% NaOH

Since the fluorescent degradation products of quinine have not all been isolated and identified chemically it was not possible to carry out recovery experiments for these substances as in the case of quinine. Consequently, their analytical values as determined by the present method should be looked upon with reservation until further information regarding their chemical nature is available.

The blank value obtained by extracting 0.5 cc blood or 2.5 cc 10% tissue suspension at pH 8.5 is very small. It amounts to a scale reading of 0.5 to 1.0 divisions with the maximal sensitivity of the instruments and B<sub>1</sub> PC-1 filters. Therefore the error introduced by the blank is insignificant for an amount of quinine in the blood and tissue samples greater than 1 microgram which, under the conditions just stated, would give a fluorometric reading of 25 divisions. However, after treatment with 2% NaOH the blank value for blood was found to be 6-7 times greater. Different samples of blood did not always give the same values. To a much less degree it was found to be the case for tissues rich in blood supply. In order to estimate the amount of the fluorescent ma

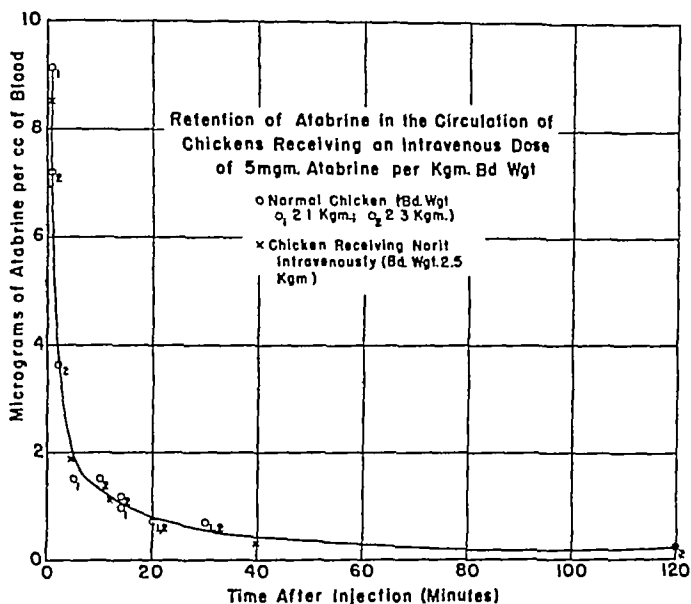


FIG 2

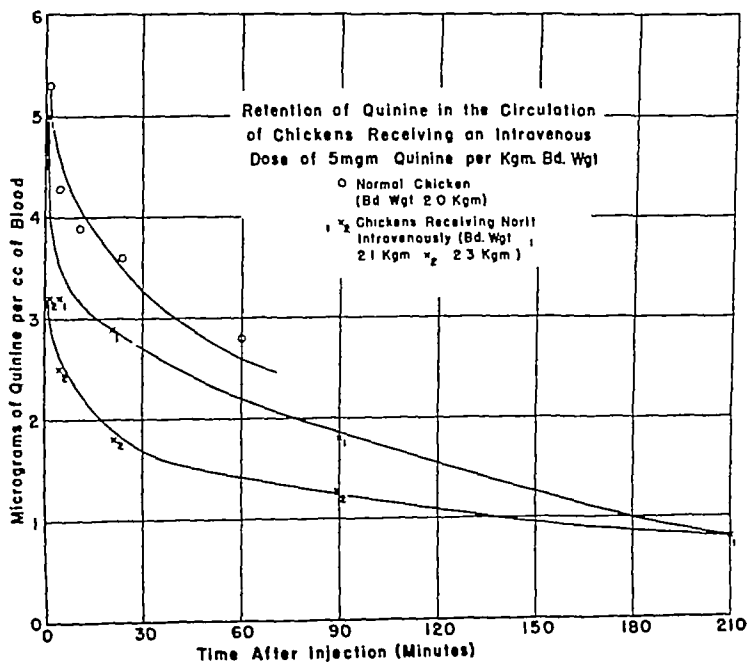


FIG 3

terial of the blood or tissues for the blank, either one of the following two procedures may be used. One is to quench the fluorescence of quinine with HCl (0.08 cc of conc HCl). The other is to isolate and determine the intensity of fluorescence of a sample first in a neutral medium i.e. 6 cc ethylene dichloride extract and 2 cc absolute alcohol, then in the presence of  $H_2SO_4$  (0.08 cc 10 N  $H_2SO_4$ ). In this neutral medium quinine does not fluoresce at all however

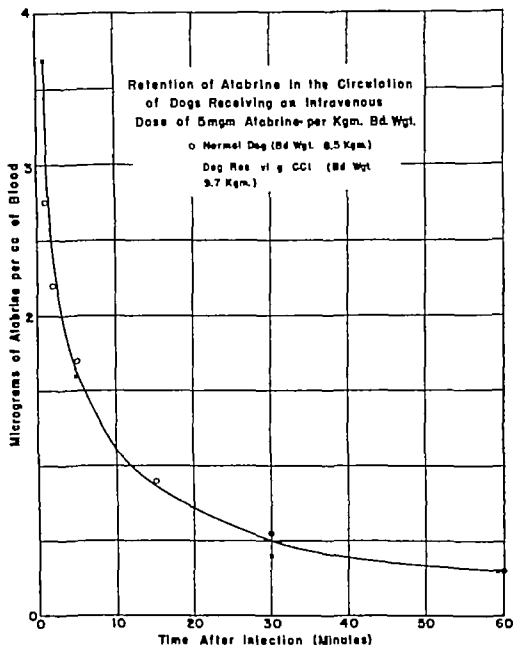


FIG 4

the intensity of fluorescence of the blood elements is greatly diminished in the presence of sulfuric acid—ten determinations of different samples of dog's blood having given an average ratio of  $2.50 \pm 0.03$ . By means of this ratio the blood blank may be determined in the same sample containing quinine while by quenching with HCl a separate sample is required. For results reported in this paper the quinine and blank determinations were made in the same sample.

The graphs in figures 2-7 were obtained by plotting the concentration of the

alkaloid in the blood on the ordinate and the time after intravenous injection on the abscissa. They indicate that both atabrine and quinine left the circulation very rapidly and reached a more or less constant low blood level one hour following the injection. The initial fall of the blood concentration of atabrine is somewhat greater than that of quinine. Partial blockage of the reticulo-endothelial system, hepatic damage, and evisceration did not in the least alter the rate of disappearance of these alkaloids from the circulation. They also disappeared from the circulation of a heart-lung preparation as quickly as from that

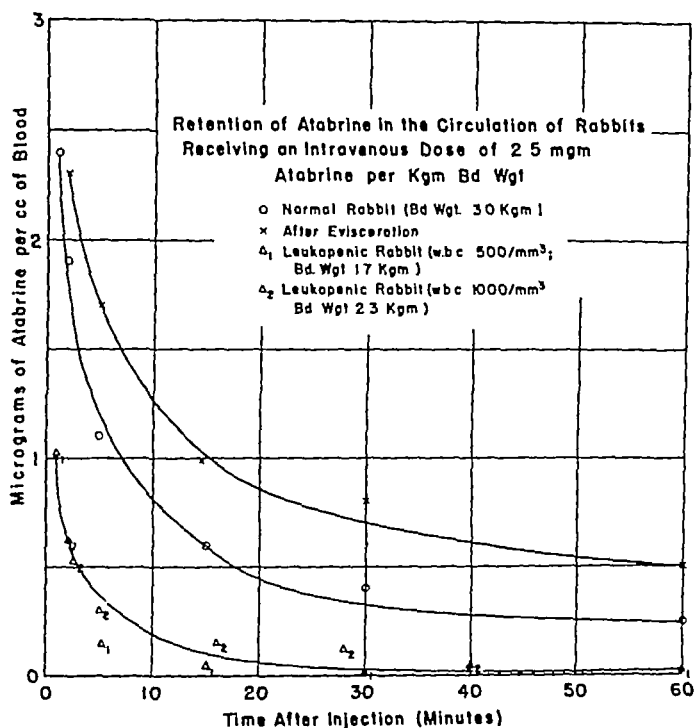


FIG 5

of normal animals. The reticulo-endothelial system or the blood elements of the bone marrow, evidently, is not involved in the initial elimination mechanism, although they may take part in the elimination process later. There was a greater drop of atabrine level in the blood of the leukopenic rabbits than that of normal animals (figure 5). The difference might have been due entirely to the diminution of the white cells which possess a greater carrying or fixing capacity for atabrine than do other constituents of the blood (9).

Considering the total volume of blood of an animal as 10 per cent of its body weight, 95 per cent of the injected atabrine or quinine disappeared from

the circulation 1-3 minutes following intravenous injection in chickens, rabbits and dogs. A value of the same magnitude was obtained in heart-lung preparations where the amount of the circulating blood could be accurately determined (fig. 7)

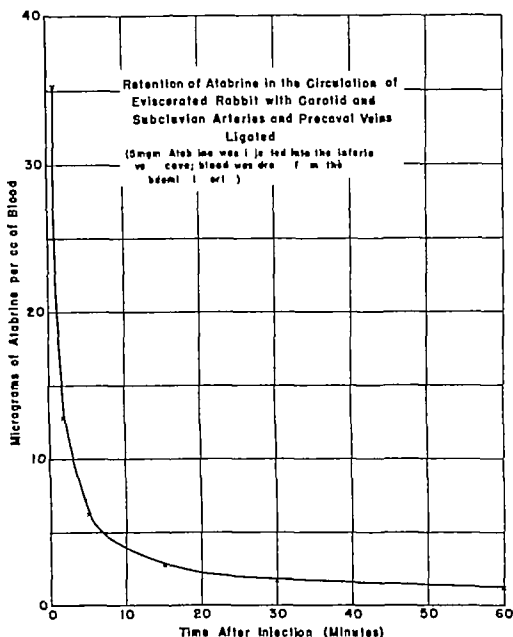


FIG 6

In normal chickens and rabbits receiving 5 mgm /kgm quinine-HCl intravenously 10-20 per cent of the quinine-like fluorescent materials in the circulation at the end of one hour was found to be due to quinine degradation products the larger quantity being in the blood of the rabbit (fig. 8)

Results of tissue analyses of heart lung preparations of the rabbit indicate that about 80-90 per cent of the injected atabrine was found in the lung the remaining 10-20 per cent in the blood and the heart. These same values were

obtained either 2 minutes or 2 hours following the injection of the alkaloid (table 2). A yellow coloration of the surface of the lung may be observed immediately after the injection. Apparently, atabrine was not easily metabolized in these tissues.

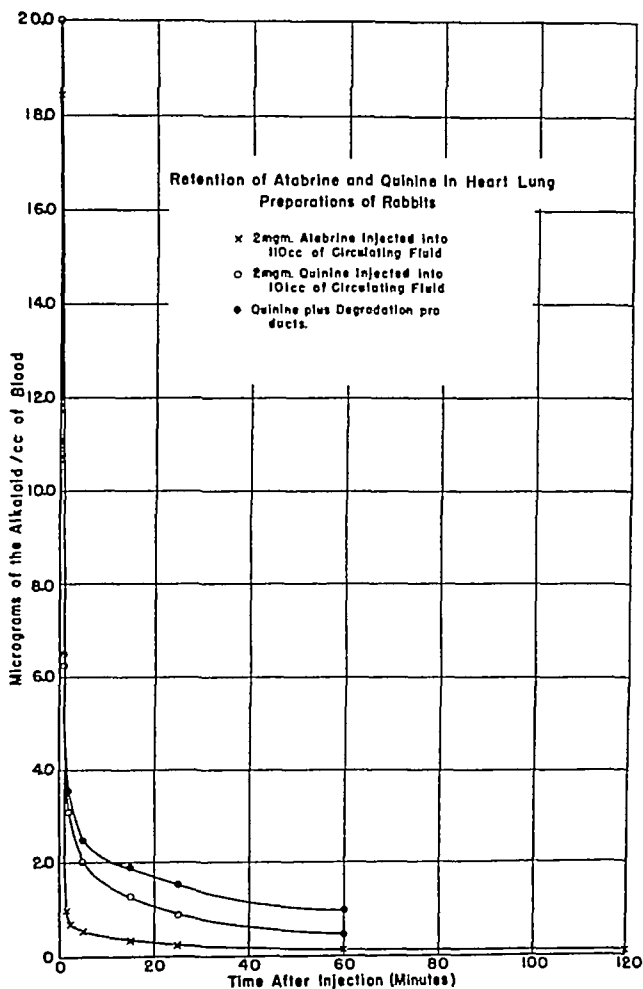


FIG 7

In the case of quinine, 20 per cent and 13 per cent of the injected alkaloid were found in the lung and in the heart and blood respectively 5 minutes following injection. After an hour, only 7 per cent of the injected quinine was recovered in the entire preparation,  $\frac{1}{3}$  of it being in the lung (table 3). With the

diminution of the quantity of quinine, there was a corresponding increase in the quantity of its fluorescent degradation products in the lung heart and blood—about 30–60 per cent of the total fluorescent materials 60–90 minutes after the injection. Evidently quinine was destroyed enzymatically very rapidly in the lung.

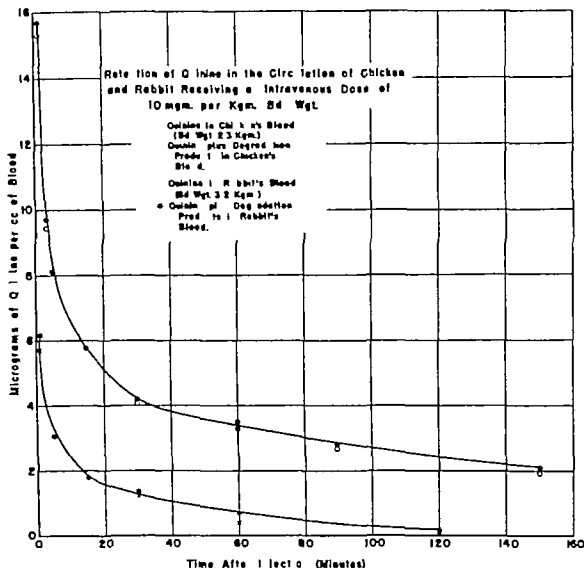


FIG. 8

The rate of this enzymatic process may be estimated from the data obtained from an experiment with lung suspensions, in which ten grams of fresh rabbit's lung were homogenized with 100 cc of 1 mgm per cent quinine-HCl in saline for 10 minutes at room temperature. During this period 80 per cent of the added quinine was enzymatically destroyed. The degradation products accounted for  $\frac{1}{3}$  of the quinine-like fluorescent substances. Boiled lung suspension was used in control experiments (table 4).

When the blood level of the alkaloid during the early stage following injection was subtracted from that at the end of one hour and this difference in

TABLE 2

*Amounts of Atabrine in the blood, heart and lung of heart lung preparations of rabbits*

EXP NO	AMT OF ATAB INJECTED	DURATION OF EXP	TISSUES	TOTAL AMT OF ATABRINE FOUND	PER CENT OF THE INJECTED ATABRINE
	mgm	min		mgm.	
1	2 0	2	Blood	0 296	14 8
			Heart	0 144	7 2
			Lung	1 548	77 4
2	2 0	90	Blood	0 046	2 3
			Heart	0 275	13 8
			Lung	1 765	88 3
3	2 0	120	Blood	0 020	1 0
			Heart	0 103	5 4
			Lung	1 849	92 5

TABLE 3

*Amounts of quinine and Q D P (quinine degradation products) in the blood, heart and lung of heart lung preparations of rabbits*

EXP NO	AMT OF QUININE INJECTED	DURATION OF EXP	TISSUES	TOTAL AMOUNT OF		PER CENT OF THE INJECTED QUININE
				Quinine + Q.D.P.	Quinine	
	mgm	min		mgm	mgm	
1	2 0	5	Blood	0 095	0 092	4 6
			Heart	0 192	0 171	8 6
			Lung	0 490	0 387	19 4
2	2 0	60	Blood	0 097	0 057	2 9
			Heart	0 059	0 040	2 0
			Lung	0 118	0 046	2 3
3	3 0	90	Blood	0 385	0 115	3 8
			Heart	0 086	0 044	1 5
			Lung	0 225	0 079	2 6

TABLE 4

*Enzymatic destruction of quinine in lung-suspension (rabbit)  
(10 minutes)*

SAMPLE (100 CC. 10% SUSPENSION)	AMOUNT OF QUININE (MGM.)			AMOUNT OF Q.D.P. † (MGM. IN TERMS OF QUININE)
	Added	After 10 minutes		
		pH 8.5	2% NaOH	
Fresh	1 0	0 309	0 208	0 101
Boiled	1 0	0 942	0 941	

\* pH 8.5, extracted at pH 8.5, 2% NaOH, digested and extracted in 2% NaOH

† Q.D.P. = quinine degradation products

blood levels and the corresponding times were plotted in their logarithms they bore a linear relationship. The lines in figure 9 were obtained by plotting the data in figures 2-7. The log/log linear relationship suggests the possibility that the mechanism involved in the initial rapid removal of the alkaloid from the circulation is an adsorption process in the tissues. In view of the overwhelmingly large quantity of the alkaloid found in the lung and the anatomical structure of this organ, the seat of the adsorption process in removing atabrine and quinine from the circulation is the lung capillaries. This supports the view

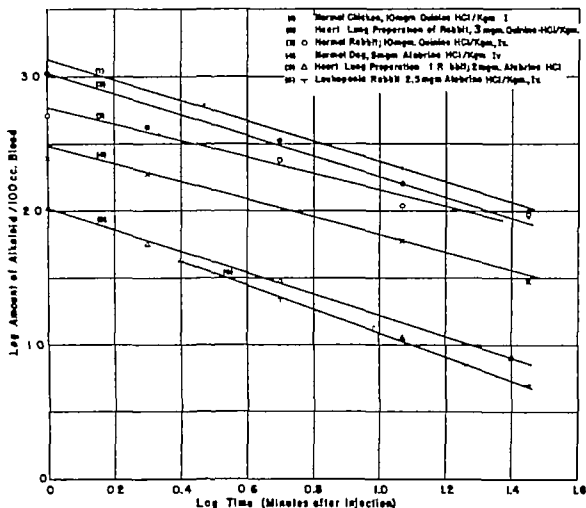


FIG 9

of Hatcher and Weiss that quinine is quickly fixed by the capillaries, it also explains the finding of Hecht that atabrine is taken up by the organs with which it first comes into contact.

#### SUMMARY

A simple rapid and accurate procedure was devised for the determination of quinine and its fluorescent degradation products in blood and tissues.

The rate at which atabrine and quinine leaves the circulation following intravenous injection has been determined in chickens after partial blockage of

the reticulo-endothelial system, in dogs with hepatic injury, in rabbits with leukopenia, and following evisceration, and in heart-lung preparations. In all cases, the alkaloid disappeared rapidly as in normal animals. The seat of elimination was found to be the capillaries, principally of the lung.

In heart-lung preparations there was no indication of any destruction of the injected atabrine for at least one hour, while 70 per cent of the injected quinine was destroyed enzymatically in 5 minutes. With the diminution of the quantity of quinine, there was a correspondingly increased amount of its fluorescent degradation products in the blood and tissues.

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# OBSERVATIONS ON THE EFFECT OF HYPERVENTILATION ON THE REFLEX RESPIRATORY STIMULATING ACTION OF NICOTINE AND CYANIDE

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It is well known that the carotid body contains chemoreceptors, excitation of which results in reflex stimulation of respiration. Among the diverse conditions and agents capable of effecting such excitation are oxygen want, carbon dioxide, cyanide, sulfide, acetylcholine, nicotine and lobeline.

The respiratory response to certain of these agents is known to be conditioned by the arterial tension of carbon dioxide. Stella (1) reports that when cyanide or sulfide is injected into the common carotid artery in the early part of an apneic pause produced by artificial hyperventilation, respiratory stimulation does not occur. Stella concludes that a certain minimal tension of  $\text{CO}_2$  in the arterial blood is necessary, either for the chemoreceptors to be stimulated by the drugs used, or which seems to him more probable, for the respiratory center to be able to respond to the afferent impulses from the receptors.

That the apneic blocking of cyanide may be peripheral rather than central would seem to follow from the work of Euler, Lilljestr nd and Zotterman (2). These workers examined the thesis that oxygen want, carbon dioxide and cyanide act on the chemoreceptors by a common mechanism, namely, a change in reaction to the acid side in the sensitive cells. Using cats anesthetized with chloralose and recording the axone potentials passing over the sinus nerve, they found that respiratory stimulation from the action of oxygen want, carbon dioxide or cyanide on the chemoreceptors of the carotid body could be effectively blocked by the simultaneous intravenous injection of small amounts (0.5-2.0 ml. of 0.5 N) of ammonia, indicating that these stimuli act by increasing the hydrogen ion concentration in the receptors. In further indication of this, they noted that an increase in the oxygen saturation of the arterial blood also greatly reduced the stimulating effect of cyanides on the chemoreceptors.

On the contrary, the respiratory stimulating action of nicotine and lobeline could not be blocked by like amounts of ammonia, indicating that their site of action might be more central. Since both are well known ganglionic stimulants, it was suggested that they act on the carotid body in a way analogous to their action on sympathetic ganglia. Extending this concept, Euler and co-workers (3) found that in the atropinized cat, acetylcholine has no effect on respiration or circulation yet markedly increases the impulse frequency over the carotid sinus nerve. In view of this, it is postulated that the afferent pathways from the chemoreceptors of the carotid body are interrupted by ganglionic cells in the region of the carotid body.

Consistent with this concept are the findings of Driggs and Dumke (4). These

investigators showed that the dose of cyanide required to stimulate the chemo receptors varied directly with the oxygen tension, whereas, the threshold dose for alpha-lobeline remained unchanged

During the course of experiments on the respiratory effects of nicotine in the dog, under Dial anesthesia, we have had occasion to note that, within limits, artificial hyperventilation will block the respiratory stimulating effect of nicotine. Thus in four of the five dogs subjected to artificial hyperventilation, injections into the femoral vein of amounts of nicotine as great as 50 micrograms per kg produced no discernible stimulation of respiration. In the normally breathing dog, 20 micrograms of nicotine per kg sufficed to produce marked stimulation. In the fifth dog it was not possible to completely block respiratory stimulation by this means, but the amount of stimulation was greatly reduced. In four other dogs under similar conditions, injection of 6 micrograms of nicotine into the common carotid artery likewise failed to stimulate respiration, whereas, in the normally breathing dog even smaller amounts sufficed.

In view of the possible theoretical bearing of our finding with nicotine and Stella's (1) finding with cyanide on the thesis proposed by Euler and co-workers (2, 3), determination of the site of block of the respiratory stimulating effects of nicotine and cyanide by artificial hyperventilation was undertaken. Assuming that the effect of artificial hyperventilation on the hydrogen ion concentration in the carotid body is qualitatively similar to that produced by injection of ammonia, then according to the concept of Euler and co-workers the site of nicotine block should be central, and the primary site of cyanide block may be peripheral.

**EXPERIMENTAL** Large dogs (15-20 kg) under Dial anesthesia were used. Blood coagulation was prevented by intravenous injection of Chlorazol Fast Pink B (2 ml of 8 per cent solution per kg). The dogs were used in pairs and the right carotid artery of one dog and the left carotid artery of another dog were ligated. The portion of the carotid of each dog peripheral to the ligation was then anastomosed by glass cannulae to the carotid of the other dog central to the ligation. The cannulae apertures were of maximum possible size so as to insure minimum changes in blood flow. Thus a carotid flow from each dog perfused a carotid sinus of the other, making it possible for each dog to act both as recipient and donor. Artificial hyperventilation of either of the dogs should result in central acapnia in the same dog and acapnia of the perfused carotid sinus in the other dog, while leaving the blood passing through the perfused carotid in the dog receiving hyperventilation unaffected. Adequacy of the hyperventilation was judged by the appearance of a substantial period of apnea in the hyperventilated dog on cessation of hyperventilation. Injections of nicotine and cyanide into the perfused carotids were made through the rubber connections between the cannulae. Nicotine doses used varied from 2-6 micrograms and sodium cyanide doses from 12-25 micrograms, depending upon the sensitivity of the animals prior to hyperventilation.

Under the above conditions, failure of nicotine or cyanide to produce reflex respiratory stimulation when injected into the perfused carotid of the dog being

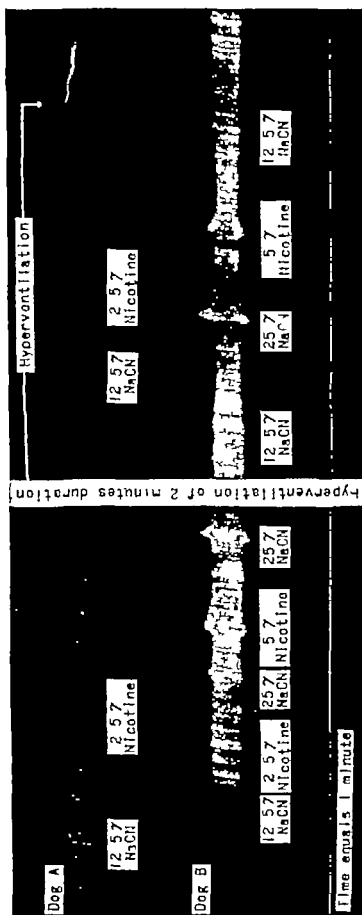


FIG. 1. Dog A, hyperventilated with the exception of one carotid blood flow coming from dog B. Dog B, normal with the exception of one carotid blood flow (hyperventilated) coming from dog A. Hyperventilation was started during the break in the record.

actively hyperventilated was interpreted as indicating central block. Similarly, failure of these substances to produce reflex respiratory stimulation when injected into the perfused carotid of the dog not being actively hyperventilated was interpreted as indicating peripheral block.

**RESULTS** In six experiments, central block of the respiratory stimulating actions of nicotine and cyanide was obtained in all cases. In only one animal was there any evidence of peripheral block to cyanide and in none did this occur to nicotine. Typical of the results are those shown in figure 1.

In the one dog showing evidence of peripheral block to cyanide, block was repeatedly obtained to 12 microgram doses of sodium cyanide but not to 25 microgram doses. Control injections of 12 microgram doses before and after hyperventilation produced definite respiratory stimulation.

#### CONCLUSIONS

1 Artificial hyperventilation of sufficient degree usually abolishes the reflex respiratory stimulating effects of nicotine and cyanide.

2 In the case of nicotine, this appears to be due to decreased responsiveness of the respiratory center to afferent impulses.

3 The results with cyanide seem best explained on a similar basis, although there is some evidence that, to a much lesser extent, depression of sensitivity of the chemoreceptors in the carotid body may occur.

4 These results essentially add no support to, but neither should they be construed as necessarily denying, the mechanism of afferent innervation of the carotid body proposed by Euler, Liljestrand and Zotterman. The possibility remains that hyperventilation apnea is an insufficient means of preventing cyanide from causing a change in reaction to the acid side at the site of the chemoreceptors.

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# THE SPONTANEOUS DEVELOPMENT OF ARSENIC-RESISTANCE IN TRYPANOSOMA EQUIPERDUM, AND ITS MECHANISM

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The development of increased resistance on the part of certain micro-organisms to the action of chemotherapeutic agents has been of considerable laboratory and clinical interest since the early days of chemotherapy. Browning, Franke, and Roehl (1) working in Ehrlich's laboratory observed that trypanosomes could be made resistant to paraformaldehyde. It has since been established that trypanosomes may become resistant to a variety of dyes, arsenicals, and antimonials (2-11), and that certain bacteria may become resistant to sulfonamides (12). The evidence is conflicting as to whether *S. pallida* may become similarly "arsenic-fast." These drug-resistant strains have been produced in the laboratory by repeated exposure of the organisms to sublethal concentrations of the same or related drugs, and when such strains appear in clinical practice it is sometimes assumed that there has been a recent similar exposure to the drug, though not necessarily in the treatment-resistant patient himself.

As was indicated by Yorke (3) it is an open question whether the production of an arsenic-fast strain of trypanosomes by repeated exposure to increasing concentrations of the drug is an obvious example of selection in a population initially of varying susceptibility, or whether the drug stimulates a true genetic mutation. Yorke himself inclined to the latter view. The following report is of interest in this connection, since we have observed the spontaneous development of a strain of *T. equiperdum* resistant to amino- and amide-substituted phenyl arsenoxides. This resistance developed in a stock strain within a relatively short period in the absence of exposure to drugs of any type, and persisted for at least 30 passages through mice and two passages through rabbits.

**METHODS AND MATERIALS.** The strain of *T. equiperdum* which has been used in this laboratory since 1940 for *in vitro* and *in vivo* assays of arsenicals was originally obtained from Dr. A. L. Tatum, of the University of Wisconsin. It was maintained in white rats and mice by the intraperitoneal inoculation of infected blood at 2 to 3 day intervals. The inoculum in mice consisted of approximately  $10^4$ - $10^5$  organisms. At irregular intervals the strain was passed through rabbits by the intravenous injection of  $10^7$  organisms.

The method used for the *in vitro* assay of trypanocidal activity, by which the change in the organisms was detected, has been previously described (13) as has the micro-technic of arsenic analysis (14).

**EXPERIMENTAL.** *A Demonstration of Arsenic Resistance in a Strain of T. equiperdum.* In October, 1943, it became apparent that certain phenyl arsen-

oxides were not as effective against trypanosomes *in vitro* as they had been in earlier assays. A large number of compounds were then tested, as well as different lots of the same compound, to determine whether the observed "resistance" was due to a change in the organisms, or, as at first suspected, to a chemical change in the compounds.

These data are summarized in table 1. The trypanocidal activities there listed are relative, referred to that of the unsubstituted phenyl arsenoxide as 100. The activity of this unsubstituted compound, and of phenyl arsenoxides with substituents other than amides, amines, or their derivatives, had not changed. Essentially the same concentrations of these were required to immobilize the organisms under the conditions of the experiment as had always been the case. On the other hand, the amino- and amide-substituted compounds were now one fifth to one-two hundredth as effective as they had been three to eighteen months previously. This change was independent of the age of the compound, and different lots of the same compound, prepared at varying intervals before the test, gave similar results.

Fortunately, some compounds had been assayed repeatedly between July, 1942, and October, 1943, making it possible to determine in retrospect when, and how rapidly, the organisms had changed in their susceptibility. These data are included in table 1 and are represented graphically in fig. 1. While there had been slight variations in trypanocidal activity between July 1942, and July, 1943, these changes were insignificant in comparison with the precipitous drop which occurred between July, 1943, and October, 1943.

A similar course of events has been observed in resistant strains of *T. rhodesiense* developed by repeated exposure to drugs (6). However, only untreated animals had been used for the propagation of the present strain since its receipt by us in 1940. Moreover, none of the mice or rats in the colony had received treatment of any kind between July, 1943, and October, 1943, since *in vivo* assays of toxicity and therapeutic activity had been suspended during the hot summer months. These circumstances make it unlikely that the trypanosomes had been exposed to arsenicals or any other drug during this period.

That the observed inactivity of amino- and amide-substituted phenyl arsenoxides and derivatives of these compounds was due to an actual change in the susceptibility of the organisms, and not to a change in the compounds, is conclusively shown by several distinct lines of evidence.

(1) As is indicated in table 1, different lots of the same compound, prepared at different times, proved identically inactive.

(2) Other types of substituted phenyl arsenoxides, similarly stored, and including highly active as well as weakly active compounds, showed no change in activity.

(3) The compounds inactive against the altered strain of trypanosome proved fully active when tested against *S. pallida* (15) (table 2).

(4) When a new strain of *T. equiperdum* was obtained from Dr. M. V. Veldee of the National Institute of Health, the trypanocidal activities of the amino- and amide-substituted compounds tested with the new strain were identical.

TABLE 1

The spontaneous development of arsenic resistance in a laboratory strain of *T. equiperdum*

TYPE OF SUBSTITUENT	COMPOUND (ALL COMPOUNDS ARE PHENYL ARSENOXIDES UNLESS OTHERWISE STATED)	TRYPAKOCIDAL ACTIVITY <sup>†</sup> VS. LABORATORY STRAIN OF <i>T. EQUIPERDUM</i> ON						TRYPAKOCIDAL ACTIVITY VS. N.I.H. STRAIN <sup>‡</sup> NOV. 1943 BY REQ.	RESISTANT FACTOR <sup>§</sup>
		July- Sept. 1942	Jan. March 1943	April June 1943	July <sup>¶</sup> 1943	Active (% of individual lots)	Mean		
Amides and substituted amides	2-NH <sub>2</sub> -4-CONH <sub>2</sub> -				31	0.28 0.16	0.19	29.0	203
	p-SO <sub>2</sub> NH <sub>2</sub> -	15		24		0.12 0.03*	0.11	21	185
	p-CONH <sub>2</sub> -	23		20		0.28 0.20* 0.25* 0.19* 0.2*	0.28	26.0	160
	p-CONHC <sub>2</sub> H <sub>4</sub> OH-	26			22	0.17* 0.14*	0.16	21	163
	p-NHCONH <sub>2</sub> -	20		6.5		0.23* 0.44	0.30	24	87
	p-SO <sub>2</sub> NHC <sub>2</sub> H <sub>4</sub> OH-				2.4	0.12* 0.08	0.19	4.9	90
	p-CONHC <sub>2</sub> H <sub>4</sub> CN-						0.22*	13.2	49
	p-CONHC <sub>2</sub> H <sub>4</sub> CONH <sub>2</sub> -	9.0		2.2		0.7* 0.24* 0.27* 0.3	0.4	8.9	22
	m-CONH <sub>2</sub> -						1.4	21	22
	p-CONHC <sub>2</sub> H <sub>4</sub> CONH <sub>2</sub> -	21			20	2.0 0.80*	1.6	25	13
	p-OC <sub>2</sub> H <sub>5</sub> CONH <sub>2</sub> -			17		1.4 1.5	1.4	17.7*	12
	p-(CH <sub>3</sub> ) <sub>2</sub> CONH <sub>2</sub> -	40.0		20*			5.4	26	7
Amines and substituted amines	2-NH <sub>2</sub> -4-OC <sub>2</sub> H <sub>4</sub> OH-						0.21	22	120
	p-CH <sub>3</sub> NHCOCH <sub>3</sub> -	27.0				0.23 0.44	0.22	22.0	23
	p-NHCOCH <sub>3</sub> -	27*		26			1.1	43	23
	2-NH <sub>2</sub> -4-OH-	20		16			0.6	21.0	24
	2-NHCOCH <sub>3</sub> -4-OH-		1.8				0.3		6.0
	p-NH <sub>2</sub> -	44			21		2.6	29*	4.8

Indicates single assay. All other values represent averages of from five to seven assays.

<sup>†</sup> Trypanocidal activity per gram of compound referred to unsubstituted phenyl arsenoxide as 100.<sup>‡</sup> N.I.H. strain = Strain of *T. equiperdum* obtained from Dr. M. V. Valdes of the National Institute of Health.<sup>§</sup> Resistance factor = Activity vs. normal strain/Activity vs. resistant strain (October 1942) = Trypanocidal concentration for resistant strain/Trypanocidal concentration for normal strain (2).<sup>¶</sup> By an unfortunate chance only one of the amines or amides listed in the table was tested during the months of August or September; for the others there are no data between July 28 and October 7. Judged by that one compound, however, arsenic-resistance was apparently almost fully established by August 20 (table 4).

TABLE 1—Continued

TYPE OF SUBSTITUENT	COMPOUND (ALL COMPOUNDS ARE PHENYL ARSENOXIDES UNLESS OTHERWISE STATED)	TRYPANOCIDAL ACTIVITY <sup>1</sup> VS LABORATORY STRAIN OF T EQUIPERDUM ON					TRYPANOCI DAL ACTIVITY VS N.I.R. STRAIN <sup>2</sup> NOV 1943 ET SEQ	RESISTANT FACTOR <sup>3</sup>	
		July- Sept. 1942	Jan - March 1943	April- June 1943	July <sup>4</sup> 1943	October 1943			
						Acti- vity of indi- vidual lots	Mean		
Acids	3-NH <sub>2</sub> -4-COOH--	29			27*		47*	3	0.7
	p-(CH <sub>3</sub> ) <sub>2</sub> COOH--	30			35		38*	33*	1.0
	p-(CH <sub>3</sub> ) <sub>3</sub> COOH--						45	44	1.0
	p-CHOHCOOH--				0.5*		0.25	0.14	1±
"Indifferent" substitu- ents	p-CH <sub>3</sub> --	92					65	82	1.3
	1 naphthylarsenoxide				60		57.5	57	1.0
	o-Cl--				75		81*	51	0.6-1.0

with those obtained with the old strain before the change in resistance, and five to two hundred times greater than their activity against the resistant strain simultaneously tested.

Repeated passages through mice, two passages through rabbits, and freezing at  $-80^{\circ}$  centigrade did not cause the resistant strain to revert to normal.

The resistance to arsenicals observed *in vitro* was also observed *in vivo*. With the original strain of *T. equiperdum*, 1.6 mg per kg of the 3-NH<sub>2</sub>-4-OH phenyl arsenoxide (mapharsen), or 1.7 mg per kg of the 3-NH<sub>2</sub>-4-CONH<sub>2</sub> compound, cured 50 per cent of infected mice (13). After the strain had become resistant, even 40 mg per kg, a dose which was in excess of the maximal tolerated dose of these two compounds, and which approached their LD<sub>50</sub> values, failed to cure any animals.

**B Specificity of Arsenic-Resistance** Yorke, Murgatroyd and Hawking (6) have pointed out that the resistance of trypanosomes to arsenicals can be highly selective, and they developed two distinct types of arsenic-resistant *T. rhodesiense*. One strain was made moderately resistant to arsenophenylglycine. In a second strain a high degree of resistance was developed to amino- and amide-substituted phenyl arsenoxides and their derivatives. The latter strain was, however, not resistant to acid-substituted arsenoxides (2), to the unsubstituted phenyl arsenoxide (8) or to phenyl arsenoxides with substituent groups such as —CH<sub>3</sub> or —NO<sub>2</sub> (2), groups which do not significantly affect the toxicity or activity of the parent compound (12).

As shown in table 3, the resistant strain of *T. equiperdum* here described closely resembled the latter resistant strain of *T. rhodesiense*, in that the resistance was primarily against the amino- and amide-substituted phenyl arsenoxides and their derivatives, with little or no resistance to the unsubstituted phenyl arsenoxide, to derivatives with "indifferent" substituents such as —CH<sub>3</sub>,

or —Cl, which affect neither activity nor toxicity, or to derivatives with acidic substituents

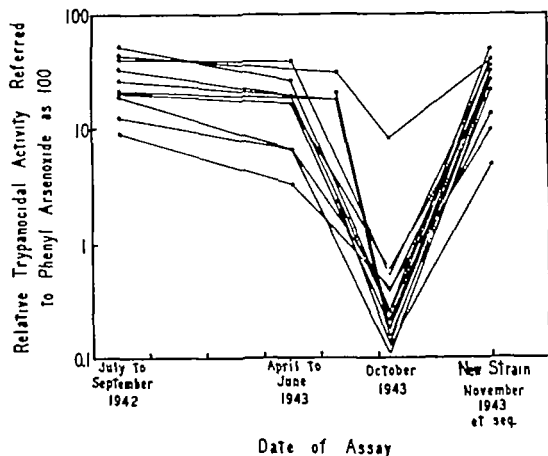


FIG 1 THE SPONTANEOUS DEVELOPMENT BY *T. EQUIPERDUM* OF RESISTANCE TO AMINO- AND AMIDE-SUBSTITUTED PHENYL ARSENOXIDES

TABLE 2

Showing that compounds against which trypanosomes were resistant (table 1) were fully active when tested against *S. pallida* (14)

COMPOUND	DATE TESTED	TRYPANOCIDAL ACTIVITY*
p-CONH <sub>2</sub> phenyl arsenoxide	June 1939	32.7
	October 1943	25
p-NHCONH <sub>2</sub> phenyl arsenoxide	May 1939	26.1
	October 1943	26.0
p-OCH <sub>3</sub> CONH <sub>2</sub> phenyl arsenoxide	September 1939	33.5
	October 1943	35

\* Per gram compound relative to that of phenyl arsenoxide as 100

The maintained activity of the acid-substituted phenyl arsenoxides against these resistant strains is of particular interest. It has been shown in a previous communication from this laboratory (13) that the p-(CH<sub>2</sub>)<sub>2</sub>COOH phenyl

arsenoxide provides an exception to the general rule that acidic substituents inhibit trypanocidal activity. This compound was highly active *in vitro*, gave a better chemotherapeutic index in experimental trypanosomiasis of mice and rabbits than any other arsenical tested, and is now under field trial in man.

TABLE 3

Comparison of "arsenic resistant" strains of *T. equiperdum* (spontaneous) and *T. rhodesiense* (induced) in their susceptibility to various types of phenyl arsenoxides

TYPE OF SUBSTITUENT	COMPOUND (ALL COMPOUNDS ARE PHENYL ARSENOXIDES UNLESS OTHERWISE STATED)	RESISTANT FACTOR	
		<i>T. equiperdum</i> (spontaneously resistant)	<i>T. rhodesiense</i> (induced resistance)
Unsubstituted phenyl arsenoxide		1 0	0 5† 1 0‡
Amides and substituted amides	p CONH <sub>2</sub> —	140	64†
	p-SO <sub>2</sub> NH <sub>2</sub> —	165	
	p-NHCH <sub>2</sub> CONH <sub>2</sub> —		256-512‡
	p NHCONH <sub>2</sub> —	57	
Amines and substituted amines	p NH <sub>2</sub> —	4-6	4-8†
	p NHCOCH <sub>3</sub> —	38	32†
	3 NH <sub>2</sub> -4-OH—	35	32†
Acids	p (CH <sub>3</sub> ) <sub>2</sub> COOH—	1 0	
	p (CH <sub>3</sub> ) <sub>2</sub> COOH—	1 0	
	p COOH—		0 5†
	3 CH <sub>2</sub> CONH <sub>2</sub> -4-COOH—		0 5†
	4 CH <sub>2</sub> CONH-3 COOH—		1 0†
	2,4 COOH—		1 0†
"Indifferent" substituents (no effect on toxicity)	p NO <sub>2</sub> —		0 5-1 0†
	2,5 CH <sub>3</sub> —		0 5-1 0†
	p CH <sub>3</sub> —	1 3	
	1 naphthyl arsenoxide—	1 0	
	o Cl—	1 0	

\* Resistant factor = activity vs normal strain/activity vs resistant strain = trypanocidal concentration for resistant strain/trypanocidal concentration for normal strain (8)

† For complete list of compounds tested with this strain see table 1

‡ King and Strangeways (2)

§ Hawking (8)

The fact that it is fully active against some resistant strains of trypanosomes extends its possible usefulness.

C. *Arsenic Resistance in Trypanosomes as a Function of a Decreased Power to Combine with Arsenicals, and Possible Explanations for the Latter*. The reason for the demonstrated resistance of this and other strains of trypanosomes to some arsenicals, but not to all, is of considerable interest. Two points of view have been expressed by previous workers, one, that the resistant organisms actually have an increased tolerance for the drug (10, 11, 16), and the second,

that the organisms are normally susceptible to bound arsenic and that their selective resistance to some arsenicals is due to their failure to combine with those specific compounds (3, 7, 8). As summarized in the following paragraphs, the weight of evidence seems to be in favor of the latter view.

With a biological method of assaying arsenical, Yorke, Murgatroyd and Hawking (7) showed that arsenic resistant strains of *T. rhodesiense* bound much less arsenic than normal strains from solutions of compounds to which they were resistant. This was confirmed by Hawking (8), with a chemical rather than biological method of assay. On the other hand Reiner, Leonard and Chao (10) and Pedlow and Reiner (11) found not enough difference between the amounts of arsenic bound by normal and resistant strains of trypanosome to account for their widely varying susceptibility and inclined to the view that the larger amounts of arsenic bound by normal trypanosomes were due to the fact that a significant portion had been damaged or killed by the arsenical. Hawking (8), however, subsequently demonstrated that the difference in combining affinity was apparent even under conditions in which the normal organisms had not been killed.

In our own experiments, the combining affinity of *T. equiperdum* for five arsenicals had by chance been tested just before and just after the strain became arsenic 'resistant' (table 4). In the case of two of those compounds, the  $p\text{-SO}_2\text{NH}_2$  and  $p\text{-CONHCH}_2\text{CONH}_2$  phenyl arsenoxides, a high degree of resistance was apparent in October, 1943, and at that time the trypanosomal suspension bound negligibly small amounts of these compounds in one to sixty minutes compared to the amounts bound in July or earlier, when the strain was normally susceptible. This difference was apparent in the shortest time periods tested when there had been no demonstrable effect on the motility of either strain, and must therefore reflect inherent differences in the combining affinity of the living intact organisms. (The large amounts bound after 24 hours represent fixation by dead trypanosomes not relevant to the present discussion.) Almost the maximal loss of affinity for the  $p\text{-SO}_2\text{NH}_2$  compound occurred between July 10 and August 30, with no significant change thereafter and it seems likely that the arsenic-resistant properties of the strain were almost fully established by the latter date.

In contrast to these results with amide-substituted compounds, in the case of three other arsenoxides to which the 'resistant' trypanosomes remained fully susceptible (the unsubstituted phenyl arsenoxide and its  $p\text{-(CH}_3)_2\text{COOH}$  and  $p\text{-Cl}$  derivatives), there was no difference in the degree to which the arsenic was bound before and after the development of its selective resistance (cf. table 4).

The present experiments with an arsenic-resistant strain of *T. equiperdum* thus confirm the findings of Yorke, Murgatroyd and Hawking (7) and Hawking (8) with a resistant strain of *T. rhodesiense*. The resistant organisms bound less arsenic than normal trypanosomes from those arsenicals to which they were resistant, but their affinity for phenyl arsenoxides to which they had not become resistant remained quantitatively unchanged.

It is relevant to this discussion to point out that the varying susceptibility

TABLE 4

*The decreased affinity of trypanosomes for those phenyl arsenozides to which they spontaneously developed resistance, and their maintained affinity for those compounds to which they remained normally susceptible*  
(Technic of experiments as described in heading to table 5)

	COMPOUND (R-C <sub>6</sub> H <sub>4</sub> AsO)	DATE	RELATIVE TRYPANOCIDAL ACTIVITY PER MOLE	NUMBER OF TRYPANOSOMES PER CC. X 10 <sup>6</sup>	CONCENTRATION OF ADDED ARSENIC (MICROGRAMS/ CC.)	PERCENTAGE OF TOTAL ARSENIC BOUND BY TRYPANOSOMES AFTER				
						1 minute	10 minutes	4 hours	24 hours	
Compounds to which organisms devel- oped resistance	p-SO <sub>2</sub> NH <sub>2</sub> --	2/28/43 to 5/13/43	19	220-330 (6 expts)	1.66		Mean = 45			
		7/16/43		250	2.0	28.7	50.5	52.6		
		8/30/43		200	2.0	7.7	11.3	25.6	41.4	
		10/ 1/43	0.28	200	2.0	3.8				
		10/ 7/43		200	2.0		0	18.7	48.0	
Compounds to which organisms did not become resistant	p-CONHCH <sub>2</sub> CONH <sub>2</sub> --	2/28/43	14	280	1.66		38			
		5/12/43		260	1.66		39			
		7/14/43		225	1.66		37			
	p (CH <sub>3</sub> ) <sub>2</sub> COOH--	10/ 1/43	0.64	250 (3 expts)	1.66		1.8, 1.8, 1.8 Mean = 1.8			
		2/25/43 to 5/13/43	54	260-280 (3 expts)	1.66		49, 55, 54			
		7/29/43		215	1.66		42			
	p-Cl--	9/23/43	54	250	1.66		59			
		5/31/43 to 6/30/43	90	245-295 (3 expts)	1.66		59, 63, 53 Mean = 58			
		7/ 8/43		245			55, 51 70			
	Unsubstituted phenyl arsen oxide	9/23/43	80	250	1.66					
		3/12/43 to 4/12/43	100	250-280 (3 expts)	1.66		79, 77, 71			
		9/10/43	100	250	1.66		70			
		9/20/43	100	250	1.66		80			

of normal trypanosomes to different arsenicals is also a direct function of their combining power with those compounds. Reiner, Leonard and Chao (10) found this to be the case on comparing the 3-NH<sub>2</sub>-4-OH phenyl arsenoxide three arspenammes and an arsonic acid. In our own experiments the correlation between the trypanocidal activity of a large series of phenyl arsenoxides and the relative amounts of each bound under standard conditions by a suspension of *T. equiperdum*, was so marked as to suggest a definite causal relationship (table 5)

It is to be emphasized that the amount of arsenic bound bore no necessary relationship to the proportion of dead organisms at the time of the test. Under the conditions of the experiment all compounds with a molar activity of 45 or more had immobilized all the organisms while all compounds with an activity of 31 or less had had no demonstrable effect. Within each group of compounds the amount of the individual arsenical bound by the organisms nevertheless varied with their trypanocidal activity. Moreover, as will be shown in a subsequent paper the chemical removal of the arsenical from organisms already immobilized and apparently dead caused a prompt return of motility and infectiousness clear evidence that the binding of arsenicals was causally related to the observed immobilization.

In summary these several observations strongly suggest that the varying susceptibility of trypanosomes to arsenicals is determined by the varying degree to which the latter are bound by the organisms. Comparing a series of compounds those with the greatest combining power are the most active. Similarly, the decreased susceptibility of arsenic resistant strains to certain compounds is apparently a function of their diminished affinity for those specific compounds. Whether in normal or resistant strains of trypanosomes, the fact which primarily determines the direct trypanocidal activity of an arsenical is the degree to which it is bound.

It has been shown in a preceding paper (17) that the widely varying toxicity of arsenicals reflects the degree to which they are bound by the tissues. The correlation here shown between their trypanocidal activity and the amount bound by the organisms is analogous to and consistent with, those findings. Although the concepts of parasitotropism and organotropism postulated by Ehrlich, and the statement that 'chemotherapeutic agents are not active unless bound' (21) may be too inclusive as generalizations they do seem entirely valid for the arsenicals. As a group these are active precisely to the degree that they are bound by the tissues or by the parasites. Their toxic or parasitocidal action therefore probably rests on the fact that at least some of the chemical groupings reactive with arsenic (perhaps -SH (22)) are functionally essential to the cell and are rendered inactive by that combination.

The underlying cause for the selective loss of affinity for arsenicals manifested by arsenic resistant strains of trypanosomes is still obscure. Hawking (8) has suggested that the 'receptor' groups for arsenic in the resistant strain are modified in the sense that they have a diminished affinity for trivalent arsenicals carrying certain substituent groups while they maintain their affinity for e.g.

TABLE 5

*The trypanocidal activity of arsenicals in relation to their binding by normal trypanosomes*

Method One cc of the arsenical solution was added to 9 cc of a buffered suspension of *T. equiperdum*. The medium consisted of one part rabbit serum, two parts 0.85 per cent NaCl, and two parts of M/10 phosphate buffer at pH 7.4. The final arsenic concentration was 1.66 micrograms per cc. Although the final concentrations of organisms varied from  $200-300 \times 10^6$  per cc, the median was  $250 \times 10^6$ , and all the results have been recalculated to that base, assuming a linear relationship between the number of organisms and the amount of arsenic bound.

After prompt and thorough mixing the specimen was allowed to stand for 10 minutes at room temperature and then sharply centrifuged. The volume of sedimented trypanosomes per  $250 \times 10^6$  organisms averaged 0.12 cc.

COMPOUND (ALL COMPOUNDS ARE PHENYL ARSENOXIDES UNLESS OTHERWISE STATED)	RELATIVE TRYPANOCIDAL ACTIVITY IN VITRO (MOLAR, REFERRED TO PHENYL ARSENIC OXIDE AS 100)	AVERAGE PER CENT OF TOTAL ARSENIC IN TRYPANOSOMES	AVERAGE CONC. CENTRATION IN TRYPANOSOMES (MG% AS %)	AVERAGE RATIO OF TRYPANOSOME AS CONC. OR SUPERNATANT AS CONC.
p SO <sub>2</sub> H—	0.06	2.5	0.41	2.4
p CONHCH <sub>2</sub> COOH—	0.22	2.9	0.36	2.5
p-SO <sub>2</sub> NHCH <sub>2</sub> CONH <sub>2</sub> —	1.4	6.1	0.68	5.2
p CH <sub>2</sub> CONHCH <sub>2</sub> CONH <sub>2</sub> —	1.5	10	1.4	9.5
p CH=CHCOOH—	2.0	1.6	0.2	1.4
3-NHCOCH <sub>2</sub> -4-OH	3.0	18	2.1	18
p OCH <sub>2</sub> COOH—	4.5	12	1.5	11
p CH <sub>2</sub> COOH—	4.7	12	1.6	12
p-CONHCH <sub>2</sub> CONH <sub>2</sub>	15	38	5.7	53
p-OCH <sub>2</sub> CONH <sub>2</sub> —	26	52	6.8	124
3-NH <sub>2</sub> -4-OH—	27	50	6.5	87
3-OH-4-NH <sub>2</sub> —	30	45	6.9	76
p NHCOCH <sub>2</sub> NH <sub>2</sub>	31	27	3.6	32
p-SO <sub>2</sub> N(C <sub>2</sub> H <sub>5</sub> ) <sub>2</sub> —	35	52	7.4	93
p NHCONH <sub>2</sub> —	35	36	5.3	52
p-SO <sub>2</sub> NH <sub>2</sub> —	30	38	5.5	54
p CONH <sub>2</sub> —	45	57	7.7	113
3 NH <sub>2</sub> -4 CONH <sub>2</sub> —	52	54	7.6	113
p (CH <sub>2</sub> ) <sub>3</sub> COOH—	54	52	6.6	94
3 NH <sub>2</sub> -4 Cl—	59	60	8.7	133
m-OH—	66	56	8.2	119
2-OH-5-As <sub>2</sub> O azobenzene	71	78	9.6	302
1-naphthyl arsenoxide	79	80	10.4	326
2,4-diCl—	80	71	9.5	204
p Cl—	90	57	7.2	116
o CH <sub>2</sub> —	91	68	9.5	186
o Cl—	92	69	9.3	189
m-Cl—	95	81	10.3	354
Phenyl arsenoxide	100	70	9.7	224
p CH <sub>2</sub> —	102	73	10.0	224
2 naphthyl arsenoxide	105	79	10.2	324

unsubstituted phenyl arsenoxide, which acts equally on normal and resistant trypanosomes. Because of an apparent correlation between the chemothera-

peutic index of a series of arsenicals (ratio of toxicity/trypanocidal activity) and their 'resistant factor' (susceptibility of resistant strain/susceptibility of normal strain) he has further suggested that the receptors in resistant organisms more nearly resemble those of the host tissues. However in a large series of amino- and amide-substituted phenyl arsenoxides tested in that respect, we have found no correlation between the toxicity of the compounds in mice or

TABLE 6

*Showing that arsenic resistant organisms do not resemble mammalian tissue in their susceptibility to arsenicals*

a) No correlation between trypanocidal activity and toxicity

b) No correlation between chemotherapeutic index  $\frac{CD_{50}}{LD_{50}}$  and resistant factor (activity vs normal strain/activity vs resistant strain)

TYPE OF COMPOUND	COMPOUND (ALL COMPOUNDS ARE PHENYL ARSENOXIDES UNLESS OTHERWISE STATED)	MOLAR ACTIVITY AGAINST RESISTANT STRAIN	MOLAR TOXICITY	CHEMOTHERAPEUTIC INDEX $\frac{CD_{50}}{LD_{50}}$ IN MICE TRYPA-NOSOMIAE	RESISTANT FACTOR (FROM TABLE 1)
Amides and substituted amides	p-SO <sub>2</sub> NH <sub>2</sub> —	0.17	4.8	8.9	165
	3-NH <sub>2</sub> -4-CONH <sub>2</sub> —	0.26	5.6	28.3	205
	p-CONH <sub>2</sub> —	0.34	9.6	7.9	140
	p-CONHC <sub>2</sub> H <sub>4</sub> OH—	0.23	4.8	15.7	155
	p-NHCONH <sub>2</sub> —	0.56	8.1	9.8	57
	p-SO <sub>2</sub> NHC <sub>2</sub> H <sub>4</sub> OH—	0.18	4.2	3.6	50
	p-CONHCH <sub>2</sub> CN—	0.42	4.5		47
	p-CONHCH <sub>2</sub> CONH <sub>2</sub> —	0.64	3.4	16	22
	m-CONH <sub>2</sub> —	1.77	9.7		23
	p-CONHCONH <sub>2</sub> —	2.90	6.4	10.8	13
	p-OCH <sub>2</sub> CONH <sub>2</sub> —	2.16	9.0	10.7	12
	p-(CH <sub>3</sub> ) <sub>2</sub> CONH <sub>2</sub> —	8.20	13.5	9.0	7
Amines and substituted amines	3-NH <sub>2</sub> -4-OC <sub>2</sub> H <sub>4</sub> OH—	0.33	5.4		120
	p-CH <sub>2</sub> NHCOCH <sub>3</sub> —	0.40	7.3	11.5	88
	p-NHCOCH <sub>3</sub> —	1.47	21.5		33
	3-NH <sub>2</sub> -4-OH—	0.93	6.9	26.6	35
	3-NHCOCH <sub>3</sub> -4-OH—	0.50	12.4	3.5	6
	p-NH <sub>2</sub> —	11.2	57.0		4.8
Acids	3-NH <sub>2</sub> -4-COOH—	6.3	15.1		0.7
	p-(CH <sub>3</sub> ) <sub>2</sub> COOH—	57.3	8.8	20.5	1.0
	p-(CH <sub>3</sub> ) COOH—	7.3	19.5		1.0

rabbits and their trypanocidal activity against the resistant strain as Hawking's thesis would imply or between their chemotherapeutic index and their 'resistant factor' (table 6)

In this same connection King and Strangeways (2) have pointed out that the unsubstituted phenyl arsenoxide to which the arsenic fast strain is just as susceptible as the normal strain is chemically no more reactive than some of its

substituted derivatives. Thus, the velocity of its oxidation is between that of the  $p$ -NHCONH<sub>2</sub> and the  $p$ -CONH<sub>2</sub> phenyl arsenoxides (18). As an alternative hypothesis in explanation of arsenic resistance, they suggested that highly active compounds which are equally effective against normal and resistant strains of trypanosome are usually devoid of hydrophilic groups, and may be more readily transported within the cell by virtue of the resulting solubility in lipoids, while compounds which are relatively inactive, and particularly so against the resistant strain, usually have polar groups and may therefore be waylaid by adsorption on polar surfaces en route to their site of action. One must assume in such case that the resistant strain contains either more, or more actively adsorbent, polar surfaces within the cell than the normal strain. The difficulty with this theory is the experimental observation that many compounds with hydrophilic substituents are highly active (table 1). Moreover, the relatively inactive compounds are actually not bound by the trypanosome (table 5), as they would be were they waylaid by adsorption short of the site of action. Similarly, the resistant strain binds less of those arsenicals to which it is resistant, and normal amounts of those to which it is normally susceptible. Phenyl arsenoxides in general are thus active to the degree to which they are bound by the cell, and the enormous differences in activity cannot be ascribed to their varying distribution on or within the cell.

There may be a single explanation for the varying susceptibility of normal trypanosomes to different phenyl arsenoxides, and the generally decreased susceptibility of an arsenic resistant strain to e.g. amino- and amide substituted compounds. Intrinsically, all phenyl arsenoxides may be identically active, i.e., with identical affinities for the receptor groups within a given cell species. The varying activity of different compounds may be determined simply by the varying degree to which substituents other than the —AsO group affect the penetration of the cell by the compound. Similarly, a genetic or adaptative change in the cell surface could well modify the ease with which certain substituted phenyl arsenoxides pass into the interior of the cell, without, however, affecting the permeability of compounds with other types of substituent groups, and thus result in a selective arsenic resistance such as that here described.

*D. Some Genetic Aspects of Arsenic-Resistance in Trypanosomes.* As Yorke (3) has pointed out, when arsenic-resistant strains of trypanosomes are produced *in vitro* or *in vivo* by their repeated exposure to increasing concentrations of a given compound, several mechanisms seem possible. In a bacterial population, there is varying susceptibility to a given drug, and repeated exposure to the drug might permit the survival and multiplication only of those organisms with an inherently higher resistance. A second possible mechanism, and the one which Yorke considered probable, is a genetic mutation, presumably stimulated by the drug, with the production of a new genetic strain.

The present experiments suggest yet a third possibility. It is conceivable that spontaneous variations such as that here described may occur in trypanosomes more frequently than now appears to be the case. When drug resistance is apparently "induced" by repeated exposure of the organisms to a specific

arsenical the compound itself may in some instances have nothing to do with the production of the variant strain. Instead, its function may be to act as a selective factor which permits a spontaneous variant to become apparent, by killing off the susceptible organisms with which it is in competition in the host and by which it would otherwise be overgrown. An analogous course of events has been observed in many species of bacteria (19), and Lewis (20) has shown the change in such cases to be due to a similar natural selection operating on a spontaneous variation. Similarly Luria (20a) has recently concluded on the basis of mutant strains that strains of *E. coli* resistant to a bacterial virus arise in sensitive strains by mutations which occur independently of the virus, the virus acting only as a selective factor which makes those mutations apparent. No explanation can be offered for the fact that the spontaneously variant strain of *T. equiperdum* here described was apparently able to overgrow the numerically predominant parent strain without the benefit of an extraneous selective factor.

There are several points which, although inconclusive as evidence, are consistent with the above thesis. One is the fact that the resistance apparently induced by repeated exposure of an organism to a given compound is not necessarily specific for that compound but may extend to other and often chemically unrelated compounds (6). This is in keeping with the thesis that the resistance is not necessarily induced by the compound, but may in some cases reflect a spontaneous change in the organism. The particular compound is then only one of several which have the same fortuitous selective action as between the original and the variant strains.

The course of events when arsenic resistance is induced by a drug is also in keeping with the thesis of a spontaneous variation. The available data (6) (table 4) indicate that the resistance becomes maximal within a relatively short period after it first becomes demonstrable. This suggests the sudden overgrowth of the parent by the variant strain, rather than a stepwise and progressive increase in resistance as the result of selection in a population of varying susceptibility or as an adaptative change.

Whether arsenic resistance reflects a true genetic mutation, or is the type of variation (Dauermodifikation) described by Jollos (23) which persists through many generations but eventually reverts to normal, is an open question. The fact that no arsenic fast strain of trypanosome has yet been observed to revert to normal suggests that the former may be the case.

#### SUMMARY

1 There is reported the spontaneous development of a strain of *T. equiperdum* resistant to amino- and amide-substituted phenyl arsenoxides and their derivatives. The circumstances surrounding the development of this strain suggest that the change did not occur as the result of selection, but as a spontaneous variation.

2 The organisms were 5 to 200 times more resistant to amino- and amide-substituted phenyl arsenoxides than was the parent strain and the resistance

was observed *in vivo* as well as *in vitro*. Their susceptibility to the unsubstituted phenyl arsenoxide, to derivatives with substituents which affect neither activity nor toxicity (e.g.  $-\text{CH}_3$ ,  $-\text{Cl}$ ), and to acid-substituted phenyl arsenoxides, was, however, unchanged.

3 The  $p\text{-(CH}_3)_3\text{COOH}$  phenyl arsenoxide, previously described as a compound with a favorable chemotherapeutic index in experimental trypanosomiasis, may be of particular therapeutic value because of its maintained activity against this, and perhaps other strains resistant to amino- and amide-substituted compounds.

4 The resistant organisms bound far less arsenic than the parent strain from solutions of the  $p\text{-SO}_2\text{NH}_2$  or  $p\text{-CONHCH}_2\text{CONH}_2$  phenyl arsenoxide, to which they were no longer susceptible, but their affinity for unsubstituted phenyl arsenoxide and its  $p\text{-(CH}_3)_3\text{COOH}$  or  $p\text{-Cl}$  derivatives, to which they were normally susceptible, remained unchanged.

5 In the above respects the organism resembled the strain of *T. rhodesiense* described by Yorke, Murgatroyd and Hawking, and rendered arsenic-resistant by repeated exposure to arsenicals.

6 Current theories as to the underlying mechanism of this selective arsenic-resistance are discussed. It is suggested that the primary factor which determines the activity of arsenicals, whether against normal or resistant trypanosomes, is the degree to which they are bound by the organisms, and that arsenic-resistant strains are modified in the sense that the cell membrane becomes less permeable to certain arsenicals. It is further suggested that, as in the present case, in many instances this change may not be induced by the arsenical, but may occur spontaneously either as a true genetic mutation, or as a temporary variation (Dauermodifikation-Jollos). Under these circumstances the suggested function of the arsenical is to make that mutation or variation apparent by acting as a selective factor fortuitously toxic to the parent, but not to the variant, strain.

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# ACUTE AND SUBACUTE TOXICITY OF DDT (2,2,-bis(p-CHLOROPHENYL)-1,1,1-TRICHLOROETHANE) TO LABORATORY ANIMALS

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As soon as it became apparent that the synthetic compound DDT might be extensively used (1), an investigation was initiated to determine the hazard of ingestion. Since the compound is practically odorless and tasteless, reasonably large quantities could unwittingly be consumed. DDT is quite insoluble in water but soluble in oils and organic solvents. The material used in this investigation was either dissolved in corn oil or used in the dry powdered state. Several species of animals and test methods were used to give a broad picture of the pharmacologic and toxicologic actions of DDT. We were especially interested in the differences in susceptibility which were manifested both within and between species.

*Experimental Single dose acute toxicity* An attempt was made to determine the acute oral toxicity of solutions of DDT on rats, mice, guinea pigs, rabbits, and chicks. The substance was administered by a stomach tube consisting of a soft rubber catheter attached to a syringe, with the exception of the tube for mice which consisted of a blunt streamlined-tip #18 needle. The animals were all maintained on commercial complete rations and given water ad libitum. The doses were adjusted according to the weight of the animals. In table 1 are summarized the data thus obtained.

All of these doses were dissolved in corn oil and the failure to get high mortality on the higher doses appears to be due to the large volume of oil given.

Of those animals in which death did not occur, many exhibited typical signs of DDT poisoning, namely, anorexia, nervousness, sensitivity to stimuli, and fine tremors. The results in the above experiments as well as in subsequent experiments show that the action of DDT is very irregular due perhaps to irregular absorption.

Attempts were also made to determine the acute toxicity of DDT by routes of administration other than by mouth. These experiments consisted of intramuscular and intraperitoneal injections in rats, intraperitoneal injection in rabbits, and intraperitoneal and subcutaneous injections in guinea pigs and mice. Solutions in corn oil were used. Although typical and persistent signs of DDT poisoning were shown in most animals, the toxic doses were found to be higher than by the oral route.

<sup>1</sup> A portion of the funds used in this investigation was supplied by a transfer, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Division of Pharmacology of the Food and Drug Administration.

A few preliminary experiments were carried out using a suspension of DDT in gum acacia. However the quantities required to produce signs of poisoning were considerably larger than those when the material was in solution.

From these acute experiments, it is evident that relatively small doses of DDT produce intoxication in small animals, that the dosage-mortality curve is

TABLE 1  
*Dosage-mortality ratio table for acute oral toxicity*

ANIMAL	DOSE	MORTALITY RATIO
	<i>mgm./kgm.</i>	
Rats	140	2/5
	180	3/5
	220	4/5
	260	1/5
	300	9/15
Rabbits	100	0/5
	140	0/5
	180	0/5
	220	0/5
	260	0/5
	400	3/10
Chicks	100	0/5
	140	0/5
	180	0/5
	220	0/5
	260	0/5
	300	0/5
Mice	142	0/10
	200	1/10
	282	0/10
	399	0/10
	448	8/10
	502	10/10
	564	9/10
	796	9/10
Guinea pig	178	0/10
	224	3/10
	282	4/10
	355	4/10
	447	2/20
	562	3/20

flat, that is both deaths and survivals occur over a wide range of dosage that rats and mice are more sensitive to DDT in single doses than guinea pigs and rabbits and that DDT in solution produces toxicity more readily than DDT in suspension.

*Subacute feeding experiments* Since DDT is insoluble in water since it acts

irregularly in single doses, and since the effects of short term exposure in several species were desired, a number of subacute experiments were carried out. These experiments included subacute feeding to rats, paired feeding to rats, subacute feeding to guinea pigs, mice, and chicks, and forced feeding for short periods to rats, mice, and rabbits. In addition to these, other feeding experiments were carried out on dogs and monkeys, and some investigations into the mode of action of DDT were made and will be reported later.

After several trials to find a tolerated dose, four groups of rats, six males and six females in each group, were fed 0.0% (control), 0.025%, 0.05%, and 0.10% respectively of powdered DDT in the diet. White rats raised in this laboratory were placed on experiment at the time of weaning in such a way that litter mates were randomized among the four groups. Body weight and food con-

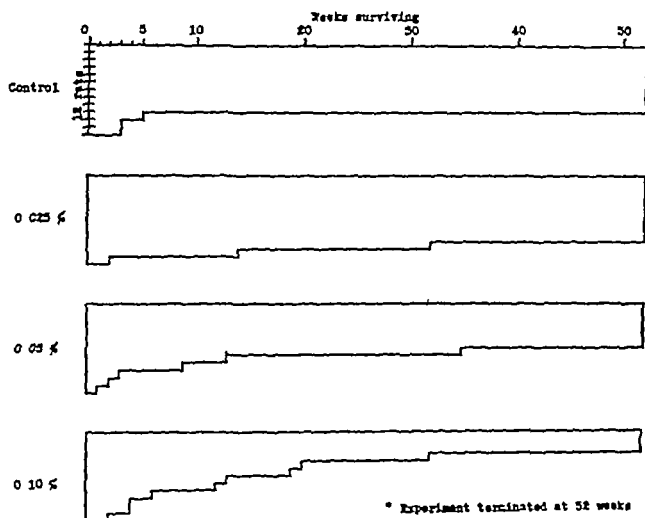


FIG 1 SURVIVAL TIMES OF RATS FED DIETS CONTAINING DDT POWDER

sumption were noted at weekly intervals. Water and food were consumed ad libitum. Several animals on the high level exhibited typical DDT symptoms after a few days, and some animals died. The remaining animals appeared to recover and except for a reduced food intake seemed quite normal. However, after varying periods of time, the rats on this level again developed nervous symptoms which grew progressively worse until the animals died. Animals dying or found dead were autopsied and tissues saved for microscopic examination. Figure 1 is a survival chart for the animals on this experiment. It will be noted that survival appears to be affected on the 0.1% and the 0.05% levels, but not on the 0.025% level. The most common pathological findings were slight to moderate liver damage which varied, appearing as focal necrosis, atrophy, hydropic degeneration or a combination of these, occasional testicular atrophy, and some degeneration in the thyroid.

In this experiment, it at first appeared as though the rats were developing a tolerance to DDT. However, when the food intake per kilogram body weight per day is calculated and plotted against age, as shown in figure 2 it is obvious that the amount of DDT consumed per kilogram per day becomes progressively less with age. This phenomenon may account for the apparent tolerance.

DDT was fed to guinea pigs in dietary levels of 0.1% and 0.05% by mixing a sufficient quantity of a 2% corn oil solution with whole commercial rabbit pellet ration. Ten guinea pigs were started on each level and ten guinea pigs were fed a control diet containing the corn oil alone mixed with the ration. The guinea pigs ate the whole pellets so that although the DDT only coated the outside of each pellet, the net result was the same as if the insecticide had been mixed throughout each pellet. The guinea pigs were of a heterogeneous

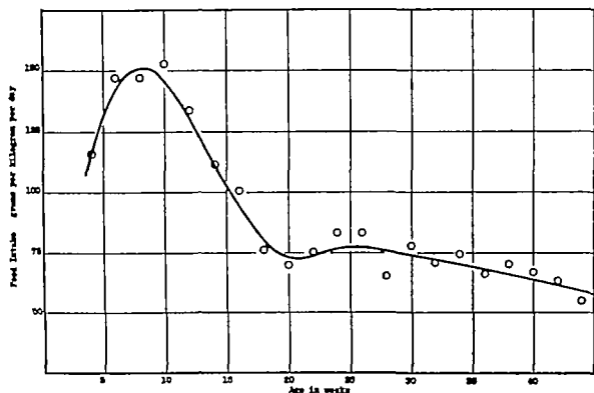


FIG. 2. CHANGE OF FOOD CONSUMPTION WITH AGE—RAT #1596

commercial supply predominantly males and were started on experiment at approximately three months of age. In table 2 is listed the survival times of these animals in weeks. The experiment was terminated after 25 weeks. The picture in the guinea pigs is partially obscured by a benign infection which occurred throughout the experimental and control groups. The infection resulted in large walled-off globular abscesses and all guinea pigs showing these were sacrificed. However the guinea pigs on the 0.1% level were definitely affected and four early deaths in the group were observed. In general the guinea pigs consumed approximately as much DDT per kilogram body weight as did the rats and therefore appeared less sensitive. The pathological lesions were similar to those found in the rats but not as striking.

It had been observed that the rats receiving DDT in the diet were hyper

irritable and often exhibited fine tremors. At the same time these animals consumed slightly less food than the controls. Therefore, a paired feeding experiment was carried out to see whether the DDT rats being more irritable used up more energy and consequently gained less weight than the controls. Eight pairs of male and eight pairs of female rats were placed on experiment at time of weaning in such a way that one member of each pair received 0.05% in the diet of DDT dissolved in corn oil mixed with the food, while the other member received a control diet to which the same amount of corn oil alone had been added. The food intake for each member of each pair was restricted to the amount that the animal eating the least would consume in a day. Neither animal was allowed to eat ad libitum. The initial and final weights are presented in table 3. The experiment was terminated after 11 weeks.

TABLE 2  
*Individual survival time of guinea pigs fed DDT*

CONTROL	0.05%	0.1%
25 wks *	25	25
25	25	25
25	24	25
20	22	25
16	20	25
15	15	21
15	14	7
15	13	3
11	13	1
6	11	1

\* Experiment terminated after 25 weeks

From these data, it does not appear that the feeding of 0.05% DDT to rats significantly slows the growth rate although the averages do fall in that direction.

Thirty chicks weighing 57 to 79 grams were divided into three groups of ten. These groups received 0.05%, 0.10%, and 0.0% (control) respectively of DDT powder mixed into a chick starting mash. In the acute toxicity experiments, the chicks had appeared quite resistant to DDT, however, all of the chicks on the 0.1% level died in from 3 to 10 days and those on the 0.05% level died in from 4 to 16 days. The microscopic pathology, a little complicated because of the fact that the diet was adequate for starting but not for growing chicks, was not striking. Many of the animals exhibited characteristic DDT tremors and in addition appeared to be unable to move voluntarily. In the initial stages of the poisoning, the experimental chicks would cower in the back of the cage while the controls would run excitedly around their cage.

Mice were fed commercial rat food biscuits coated with a corn oil solution of DDT such that each diet contained 0.05%, 0.025%, and 0.0125% DDT. However, the mice ate the more concentrated DDT coating of the pellets and con-

sequently received more than these levels of DDT. All mice fed these pellets died in from 1 to 6 days.

Rats, mice, rabbits, and guinea pigs were force-fed orally about 50% of the estimated LD50 of DDT in corn oil daily for varying periods of time. These animals were then sacrificed for microscopic examination of the tissues. A complete description of the pathological findings on all animals is reported elsewhere (2).

**DISCUSSION** DDT in relatively small doses can produce signs of toxicity in several species of animals. These signs vary in intensity with the species, with the individual animal, and with the dose of the material, but usually follow a definite pattern. The animal first becomes hyperirritable, and decreases

TABLE 3  
*Paired feeding in rats*

SEX	INITIAL WEIGHTS		FINAL WEIGHTS	
	Control	Experimental	Control	Experimental
Male	29	38	307	283
	28	38	278	281
	42	44	299	336
	38	40	325	299
	38	40		Died after 1 week
	41	39	290	290
	38	40		Died after 4 weeks
	40	42	295	285
Female	32	35	200	195
	40	41	220	210
	36	38	178	166
	40	38	190	175
	38	38	196	186
	36	36	207	215
	32	30	215	170
	38	38	194	220

Animals exhibited characteristic DDT poisoning nervous symptoms

food consumption. The hyperirritable stage progresses to fine tremors that become more marked by a stimulus such as striking the cage. After this stage the animal sometimes becomes depressed and often shows inability to carry out voluntary movements. Death usually occurs at this point, but recovery results if the animal only reaches the stage of fine tremors. The tremors are similar to those seen in phenol poisoning but can be strengthened by noise stimuli.

Characteristic also in DDT poisoning is the wide variation in individual susceptibility. Some animals appear to thrive on doses that are twice those producing death in other animals. Whenever such wide variation in susceptibility occurs it becomes very difficult to estimate the level which would only

infrequently cause signs of toxicity. The material is practically odorless and tasteless so that it becomes even more hazardous since no "warning" is given. There appears to be one safety factor, however, in the effect on the appetite. This effect is usually the first sign of toxicity.

#### SUMMARY

DDT, a synthetic insecticide, is acutely toxic by mouth to small laboratory animals (rats, mice, guinea pigs, rabbits, and chicks) in doses ranging from 150 to 750 mgm /kgm. Acute doses may produce anorexia, tremors, depression and death.

DDT is capable of causing subacute toxicity when given in small amounts in the diet for periods of from 3 days to 20 weeks. Definite signs of toxicity are produced by levels in the diet of 0.05% (500 ppm) for rats and mice, 0.1% for guinea pigs, and less than 0.05% for growing chicks.

Characteristic of DDT poisoning is the wide variation in individual susceptibility, making the estimate of a safely tolerated dose extremely difficult.

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# THE PERCUTANEOUS ABSORPTION OF DDT (2,2 BIS (p-CHLORO PHENYL) 1,1,1 TRICHLOROETHANE) IN LABORATORY ANIMALS

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Although Leidler (1) reported the synthesis of DDT as early as 1874, the knowledge of the efficacy of this compound as an insecticidal agent is the result of relatively recent entomological research (2)

The suitability of DDT preparations for cutaneous application in the treatment of lice infestations and scabies and the possible hazard of unavoidable contact in the handling of other preparations of DDT could only be established following a toxicological investigation of the skin penetration of this agent.

We have studied eighteen preparations containing DDT either as simple mixtures with bland powders, or in combination with other lousicidal agents repellents, surface active agents and various solvents. In order to restrict this paper to the toxicology of DDT following cutaneous applications only such data as pertain to DDT per se and incorporated in a bland non toxic, diluent powder as well as data on solutions of DDT in organic solvents are presented. For information concerning the toxicity and pathological findings resulting from DDT following other routes of administration, reference should be made to several papers submitted for publication (3-8)

**EXPERIMENTAL. Techniques** The toxicity studies involve four different procedures namely acute two types of subacute (3 weeks and 90 days) and sensitization experiments. The techniques involved in the individual types of experiments are given in detail elsewhere (9)

Briefly the method for the study of the acute toxicities of liquid preparations involves the use of a soft rubber cuff shaped in the form of a girdle fitted snugly around the trunk of the animal. The subject is prepared by clipping the skin free of hair in the area for exposure and in some of the animals the skin is further prepared by making epidermal abrasions longitudinally over the area 2 cm. apart. The experimental subject is placed in a comfortable but immobilized position in a special animal holder (10). The dose is administered on a body weight basis and is introduced under the cuff. The cuffs are constricted at each end and are sufficiently flared in the middle to retain the dose without pressure. Circulation of the dose around the area of exposure is thus permitted. Exposures are for periods of 24 hours following which the animals are placed in metabolism cages and observed for a minimal period of two weeks. The skin reactions during and following exposure are noted. Clinical symptoms of poisoning and blood morphological

<sup>1</sup> A portion of the funds used in this investigation was supplied by a transfer recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Division of Pharmacology of the Food and Drug Administration.

changes, if any, are recorded, and routine chemical tests on blood and urine are done. Body weights are recorded daily and the food intake noted. Severely poisoned animals are sacrificed for histological examination of their tissues.

In the case of both the short and the longer (90-day) subacute experiments the subjects are inoculated daily with given doses of the agent. The selection of the size of the dose depends on the results obtained in the acute exposures. The same general observations and tests are made as in the case of the animals in the acute exposures above. In the subacute experiments, however, emphasis is placed on the criteria which indicate gradual impairment of normal physiological action, such as special tests for liver and kidney function, changes in blood morphology, anorexia, loss in weight, etc. The tissues of all 90-day animals are examined histologically.

Tests for potential sensitization are made on the guinea pig using a slight modification of the Landsteiner technique (11).

Primary irritation is determined in the rabbit using a patch test technique on both the intact and abraded skin (9).

*Acute Experiments* The first DDT preparation submitted for toxicological study was a powder of the following composition

DDT	5%
Talc	95%

Acute toxicity tests were made on a series of six rabbits weighing approximately 3 kgm each. Each animal was exposed to a dose of 4 grams of the powder per kgm. Neither the dry nor the wetted powder (physiological saline) caused symptoms of toxicity. A very mild erythema was noted in both intact and abraded skin.

Since the 5% powder failed to produce symptoms of toxicity, undiluted DDT powder at a dosage level of 4 gms/kgm was applied to a series of six rabbits prepared in the usual manner for acute exposure. No symptoms of toxicity or of skin irritation were noted. In this exposure to DDT in the solid form, absorption was not sufficient to elicit symptoms of poisoning.

In a similar acute experiment a 10% solution of DDT in corn oil was applied at dosage levels of 390, 600 and 940 mgm of DDT/kgm. Although this solution was well absorbed these animals did not exhibit any symptoms of toxicity, but there was a mild skin erythema which persisted approximately two days beyond the period of exposure.

Since the acute experiments mentioned above failed to produce symptoms of toxicity, it was decided to determine whether or not solutions of DDT in other organic solvents might prove as innocuous. Both the dimethyl and dibutyl esters of phthalic acid are good solvents for DDT. Accordingly 30% and 25% solutions of DDT in dimethyl and dibutyl phthalates respectively, were prepared. In the acute exposures by cuff, doses of 3.9, 6.0 and 9.4 cc/kgm were employed for each solution to series of six rabbits each. In the case of the dimethyl phthalate solution, these doses represented 1.17, 1.8 and 2.82 grams of DDT/kgm, whereas with the dibutyl phthalate solutions it represented 0.975, 1.5 and 2.35 grams of DDT/kgm respectively. No deaths were obtained at any of the dosage levels, but all animals exhibited symptoms of DDT poisoning, being very severe at the 9.4 cc/kgm dosage level. In all cases the doses

were poorly absorbed, approximately 25% of the largest doses remaining in the cuff following the 24-hour exposure. The animals exhibited anorexia, body weight loss, hyperexcitability, and nervous tremors. Clonic convulsions were noted in the animals on the highest dosage levels. These latter subjects remained in very poor physical condition for several days and were sacrificed for the histological examination of their tissues.

*Short Subacute Experiment.* In this experiment, 0.5 cc of a 5% suspension of the above powder in diethylene glycol monoethyl ether (carbitol) was applied daily for three weeks to a 2.5 cm<sup>2</sup> area of the back of each of six albino rabbits. A mild erythema restricted to the area of application was noted during the second week of application. A retest application after one week had elapsed following the three-week treatment did not indicate a sensitization.

*Ninety Day Subacute Experiment.* The primary object of toxicological experiments on lousicides or insect repellents is the determination of the given agents' safety for use. The daily application by munction of a wide range of doses to various species of laboratory animals should yield information on (a) the severity and nature of the systemic poisoning, clinical manifestations, etc. (b) the degree and nature of damage in parenchymatous organs. (c) the prognosis for recovery from the damage observed, and (d) the direct effect on the skin. The proper appraisal of these factors are important aids in the determination of safety. It is the object of the longer subacute experiments to furnish the major portion of the data for the appraisal of the safety of a given compound.

The rabbit, rat, guinea pig and dog have been used for the 90-day experiments. The solution used was a 30% solution of DDT in dimethyl phthalate. The rabbits, rats and guinea pigs were inoculated at dosage levels of 150, 300, 600 and 1200 mgm. of DDT/kgm. and the dogs at levels of 300, 600 and 1200 mgm. of DDT/kgm. The results of these experiments, except the data on dogs, are summarized in tables 1, 2 and 3.

The dogs completed the 90-day experiment without exhibiting any symptoms of toxicity. The tissues of these dogs were examined histologically.

Gross pathological examination of the dogs inoculated for 90 days showed no changes attributable to the DDT. Microscopic sections were made of about 25 samples of tissue from each animal. Sudan IV stains for fat in frozen sections of the liver and kidney were made in addition to the routine paraffin sections. These sections were negative except for those of the liver and possibly those of the gall bladder. In two dogs the hepatic cells were moderately vacuolated the vacuoles being small and large, single and multiple. In one of these there was a slight, rather acute pericholangitis and very slight periportal atrophy. In the other the centrilobular hepatic cells stained less deeply than the peripheral, and contained a moderate number of cytoplasmic oxyphilic hyaline globules. Two other dogs showed minimal degrees of similar hepatic cell vacuolation; one of these also had slight centrilobular atrophy. The remaining dog showed slight hydropic change of the more usual type, with diffusely rarefied hepatic cells. Cytoplasmic hyaline globules were also present, but smaller and fewer.

than in the other dog in which they were seen Sudan IV stains on all of these livers showed the hepatic cells devoid of fat except for a very small amount in the periportal areas of one dog, this dog and one other also had small amounts of fat in the portal macrophages Mononuclear cell infiltration of the lamina

TABLE 1  
*The daily intunction of 80% DDT in dimethyl phthalate in rabbits*

RABBIT NO	SEX	WEIGHT AT START OF EXPERIMENT	DOSE IN CC. OF SOLUTION PER KG	SYMPTOMS OBSERVED	REMARKS
		grams			
1519	Male	2625	4 0	Anorexia from first dose, muscular tremors, hyper-excitability	Sacrificed after 6th dose for histological examination, moribund Weight loss of 840 grams
1520	Male	2265	4 0	Anorexia from first dose, muscular tremors, hyper-excitability	Death after 5th dose Weight loss of 550 grams
1521	Male	2780	4 0	Anorexia from first dose, muscular tremors, hyper-excitability	Death after 6th dose, weight loss of 860 grams
1522	Male	2390	2 0	Anorexia from first dose, muscular tremors, hyper-excitability	Death after 6th dose, weight loss 780 grams
1523	Female	2820	2 0	Anorexia from first dose, muscular tremors, hyper excitability	Sacrificed after 5th dose Moribund Weight loss of 705 grams
1524	Male	2400	2 0	Anorexia from first dose, muscular tremors, hyper-excitability	Sacrificed after 6th dose Moribund Weight loss of 470 grams
1525	Male	2370	1 0	Nervous tremors and mild anorexia after first dose	Survived 90 days Weight loss of 55 grams
1526	Male	2540	1 0	Nervous tremors and mild anorexia after first dose	Death after 14 doses Weight loss of 605 grams
1527	Male	2070	1 0	Nervous tremors and mild anorexia after first dose	Death after 10th dose
1528	Female	2550	0 5	Slight nervous tremors after 5th dose, slight anorexia	Survived 90-day experiment Weight gain of 725 grams
1529	Male	2680	0 5	Slight anorexia after third dose Slight nervous tremors after 5th dose	Sacrificed for histological examination after 45th dose Moribund, weight loss of 790 grams
1530	Female	2480	0 5	Slight anorexia after 5th dose	Death after 33rd dose Weight loss of 1195 grams

propria of the gall bladder was present in somewhat greater degree than ordinarily seen in our dogs, and might well have been an accompaniment of the mild liver damage Lung, heart, spleen, kidney, pancreas, lymph nodes, stomach, small intestine, colon, adrenal, genital organs, thyroid, parathyroid,

skin and (in one animal) the nervous system and voluntary muscle showed no changes attributable to DDT

By this procedure the rabbit, rat and guinea pig are approximately equally susceptible to DDT poisoning. It is evident from these experiments that wide individual variations to poisoning existed in a given animal species. Deaths

TABLE 2  
*The daily inunction of 30% DDT in dimethyl phthalate in rats*

RAT NO	SEX	DOSE CC./KGM.	WEIGHT AT BEGINNING OF EXPERIMENT	WEIGHT AT END OF EXPERIMENT OR OF PRIOR DEATH IF INDICATED	REMARKS
			GRAMS	GRAMS	
1	Male	4	255	198 at death	Severe tremors following first dose died after 10th inunction
2	Male	4	305	277 at death	Severe tremors following first dose died after 5th inunction
3	Female	4	215	160 at death	Severe tremors following 2nd dose died after 5th inunction
4	Male	2	333	240 at death	Moderate tremors during first 5 doses subsequent severe tremors to death died after 8 inunctions
5	Male	2	275	234 at death	Moderate to severe tremors after 2nd dose Died after 30 inunctions
6	Male	2	315	230 at death	Slight tremors after first inunction died after 5 inunctions
7	Male	1	290	232 survived 90-day experiment	Slight tremors after first dose exhibited severe tremors with apparent recoveries during the 90-day period
8	Male	1	345	241 at death	Slight to moderate tremors after first dose died after 49 inunctions
9	Male	1	270	315 survived 90-day experiment	Slight tremors in first week of experiment several periods of severe tremors with apparent recoveries during the 90-day experiment
10	Male	0.5	360	451 survived 90-day experiment	No symptoms of toxicity during the 90-day experiment
11	Male	0.5	275	320 survived 90-day experiment	Very slight tremors during 3rd week of experiment No symptoms after the 6th week of inunctions
12	Female	0.5	253	197 at death	Tremors following 5th inunction Died after 10th dose

occurring in these three species after 30 or more inunctions were invariably the result of secondary infections. It appears that the anorexia, and the general condition of emaciation which follows as a result of DDT intoxication seriously impairs the subject's resistance to infections.

Examination of the blood of animals at various intervals during the experiment did not reveal any serious anemias except in the subjects at the point of

death There was a moderate leukocytosis, with a definite increase in the percentage of heterophiles When a 60 per cent increase of heterophiles occurred prognosis was never favorable The report on the pathology observed in these animals is given in another paper (7)

TABLE 3

*The daily inunction of 30% DDT in dimethyl phthalate in guinea pigs*

GUINEA PIG NO	SEX	DOSE	WEIGHT AT BE- GINNING OF EX- PERIMENT	WEIGHT AT END OF EXPERIMENT OR OF PRIOR DEATH IF INDICATED	REMARKS
			cc./kgm grams	grams	
1	Male	4	371	316 at death	Slight tremors after 3rd dose, died after 4 inunctions
2	Male	4	347	294 at death	Moderate tremors following 3rd inunction, died following 5th dose
3	Male	4	320	280 at death	Slight tremors following first inunction, died after 2nd inunction
4	Female	2	340	274 at death	Slight tremors following 5th dose, died after 12 inunctions
5	Male	2	315	244 at death	Slight tremors following 9th dose, died after 29 inunctions
6	Male	2	200	285 at death	Slight tremors following 4th dose, made slight steady weight gain until 3 days prior to death, died after 44 inunctions, no symptoms of toxicity
7	Female	1	316	330 at death	No symptoms of toxicity, made slight weight gains until 3 days prior to death Died after 65 inunctions
8	Female	1	343	467 survived 90-day experiment	No symptoms of toxicity exhibited
9	Male	1	298	560 survived 90-day experiment	No symptoms of toxicity exhibited
10	Male	0.5	284	242 at death	No symptoms exhibited except slight body weight loss, died after 27 inunctions
11	Female	0.5	395	362 survived 90-day experiment	No symptoms of toxicity Made steady but slight weight gains through 10th week followed by weight loss at end of experiment
12	Female	0.5	296	293 survived 90-day experiment	Slight steady weight gains first nine weeks of experiment and steady weight loss during balance of experiment

*Sensitization Experiment* The intracutaneous injection technique in guinea pigs gave the results summarized in table 4 DDT caused a mild but definite sensitization in the guinea pig

*Primary Irritation* Tests for primary irritation were conducted on two series of 6 albino rabbits The results of the tests for the 5% DDT powder and for the 30% DDT solution in dimethyl phthalate are given in table 5

In the case of the 30% DDT solution in dimethyl phthalate a mild degree of primary irritation was obtained

Patch tests in humans and the daily contact of hands of operators with 30% solution of DDT in dimethyl phthalate produced no evidence of irritation

**DISCUSSION** The exposure to relatively large doses of undiluted DDT in powdered form produced no symptoms of poisoning in animals with either intact or abraded skin. The DDT in powdered form is poorly absorbed and in this form is non-irritating to the skin. Solutions of DDT, on the other hand in a non irritant solvent such as dimethyl phthalate were absorbed by both the intact and the abraded skin and caused severe poisoning. In acute exposures by cuff (in which the gentle pressure of inunction was not a factor) a 30% solution of DDT in dimethyl phthalate is poorly and slowly absorbed. In the sub-

TABLE 4  
*Sensitisation in guinea pigs with DDT*

FIRST READING		RETEST READING	
SUM		SUM	
0 x 8		$\frac{1}{2}$ x 20	
$\frac{1}{2}$ x 5		$\frac{1}{2}$ x 15	
0 x 0		$\frac{1}{2}$ x 15	
0 x 0		0 x 16	
0 x 0		$\frac{1}{2}$ x 15	
0 x 0		$\frac{1}{2}$ x 12	
Sum	$\frac{1}{2}$ x 8	2 $\frac{1}{2}$	x 93
Average	0.08 x 1.33	0.43	x 15.5

TABLE 5  
*Primary irritation of DDT preparations in the rabbit*

	INTACT SKIN	ABRADED SKIN	AVERAGE FOR SIX INDIVIDUALS
DDT 30% in dimethyl phthalate	2.5	5.8	4.1
DDT 5% in talc	0.0	0.8	0.4

acute experiments involving inunction the animals except the dogs receiving 1200 and 600 mgm./kgm. survived but 6 to 8 doses prior to death. On the higher dosage levels the animals exhibited an immediate severe anorexia, body weight loss, hyperexcitability and the typical nervous tremors leading to clonic convulsions. Animals surviving the lower dosage levels made apparent recoveries after early symptoms of poisoning, but became emaciated and are easy prey to secondary infections.

The guinea pig was as susceptible to fatal poisoning as the rat and rabbit, however, it did not exhibit the tremors as early in the stage of poisoning nor were the tremors as marked in these animals on the lower dosage levels. The dog was definitely less susceptible to poisoning from cutaneously applied DDT solutions.

The most pronounced change in blood elements was a moderate leukocytosis

In severely poisoned animals there was a marked increase in the percentage of heterophiles. The animals died shortly after a 60 per cent increase in heterophiles was noted.

Solutions of DDT are mildly irritating to intact and abraded skin. No irritation was noted from powdered DDT when applied by patch tests, or on the hands of operators who have had almost daily contact with it during the past year.

Various toxicological experiments with eighteen preparations of DDT indicate that DDT in the solid form appears safe for use in preparations intended for topical application to the skin. From solutions DDT is absorbed and is a severe systemic poison, however, a number of preparations submitted to us containing DDT in concentrations up to 5% have proven safe for limited use.

#### CONCLUSIONS

1 The application of powders containing 5% DDT to the skin produced no evidence of systemic toxicity or of primary irritation.

2 Solutions of DDT in dimethyl phthalate are absorbed by both intact and abraded skin.

3 Solutions of DDT cause a mild but definite sensitization in the guinea pig.

4 The munction of doses as low as 0.5 ml of a 30% solution of DDT per kgm/day (150 mgm/kgm/day of DDT) to rabbits, rats and guinea pigs may cause death in some cases after 30 days.

5 In the animal species tested there were wide individual variations in susceptibility.

6 Affected animals exhibited anorexia, severe weight loss, hyperexcitability, nervous tremors leading to clonic convulsions. Emaciated animals became easy prey to secondary infections.

7 Severely poisoned animals exhibited a moderate leukocytosis with a characteristic increase in the percentage of heterophiles.

8 The above data indicate that the unlimited use of DDT solutions on the skin is not free of danger, however, some solutions of DDT have been found safe for restricted use.

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## STUDIES ON VERATRUM ALKALOIDS

### VI PROTOVERATRINE ITS COMPARATIVE TOXICITY AND ITS CIRCULATORY ACTION<sup>1</sup>

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Protoveratrine was first isolated by Salzberger (1) from *Veratrum album*. Further chemical studies by Poethke (2, 3) revealed that it is a triacyl ester of the alkaline protoverine and that the three acids in combination with protoverine are acetic acid, methylethyl acetic acid, and methylethyl glycolic acid. According to Craig and Jacobs (4) and Jacobs and Craig (5) the empirical formulae of protoveratrine and protoverine are  $C_{27}H_{41}O_{12}N$  and  $C_{17}H_{25}O_8N$  respectively.

From the alkaloid mixture of *Veratrum album* various other alkaloids have been isolated: the ester alkaloids germerine and protoveratridine and the alkalamines jervine, rubijervine, and pseudojervine while the bulk of the crude alkaloidal material still remains to be identified (2, 3, 6). Germerine is an ester of germine and the two acids methylethyl acetic acid and methylethyl glycolic acid while protoveratridine, a degradation product of germerine (3) is an ester of germine with methylethyl acetic acid.

Jervine was first isolated from *Veratrum album* and crystallized by Simon (7) in addition to jervine Wright and Luff isolated pseudojervine and rubijervine (8). That jervine was present in *Veratrum viride* was proved by Mitchell (9) and confirmed by Wright (10) who found pseudojervine and rubijervine also in this species of *veratrum*. Seiferle (11) has recently made a chemical study of the alkaloids of *Veratrum viride* and was able to isolate for the first time protoveratridine and germine and to confirm earlier studies on jervine rubijervine and pseudojervine. His data suggest that an ester alkaloid like germerine was present, while no evidence was found for the presence of protoveratrine in his sample of *Veratrum viride*.

Like cevine and protoverine the alkalamines germine, jervine, and rubijervine are  $C_{27}$  compounds and all these alkalamines appear to be related to the solanum alkaloids, which have been recognized as sterol alkaloids (12). As far as the sterol nucleus is concerned the *veratrum* alkalamines therefore, are related to the aglucones of the cardiac glycosides.

The first pharmacological study of protoveratrine was conducted by Eden (13) using the lactate or sulfate of Salzberger's substance. He recorded the toxic effects in frogs and in normal mammals (rabbits, cats, and dogs) and the circulatory effects in these three species of mammals under ether anesthesia and curare. A comparison of the action of protoveratrine and cevadine upon the

<sup>1</sup> Part of the expense of this work was defrayed by a grant from the Ella Sachs Plots Foundation.

frog heart was made by Boehm (14), using Straub's technique. In a few experiments Haas (15) used the protoveratrine of Poethke to compare its effect with that of germerine, protoveratridine, and germine with regard to its toxicity in frogs and in rats.

The present investigation was undertaken to study the toxicity of protoveratrine in comparison with that of other chemically well-defined alkaloids of the veratrum series and especially to investigate the circulatory action of protoveratrine with a view to comparing it with veratridine in regard to such effects as the inotropic action on the heart and the action upon circulatory reflexes which only recently have been given proper attention.

TABLE 1

ALKALOID	ANALYSIS		ROTATION	MELTING POINT
	C	H		
Protoveratrine	61.56	7.94	$[\alpha]_D^{25} = -8.5^\circ$ ( $c = 1.99$ in chloroform)	Decomposes at about $273^\circ$ (uncorr.) after discoloration
Protoverine	61.45	8.3	$[\alpha]_D^{25} = -13^\circ$ ( $c = 0.86$ in pyridine recalculated for dry substance)	Softens to a resin at $195-200^\circ$
Germine	63.21	8.57	No rotation taken	Melts to a resin at $168-171^\circ$
Cevine	63.21	8.59	No rotation taken	Melts, not sharply, to a resin at $170-175^\circ$ , depending somewhat on the rate of heating
Jervine	76.02	9.34	$[\alpha]_D^{25} = -153^\circ$ ( $c = 0.99$ in ethanol recalculated for dry substance)	$236-239^\circ$
Rubijervine	78.29	10.55	$[\alpha]_D^{25} = +18^\circ$ ( $c = 1.04$ in ethanol)	$240-242^\circ$

We were fortunate in obtaining pure alkaloids of *Veratrum album* through the generosity of Dr. W. A. Jacobs (16), who put at our disposal the data assembled in table 1 concerning the properties of his substances.

The veratridine used in the toxicity tests was prepared by Linstead and Todd (17).

**METHODS** For an evaluation of the comparative toxicity white mice of both sexes weighing from 15 to 30 grams were used. Groups of six animals were injected with each dose. Injection was always made into a tail vein in a period of exactly ten seconds, and the strength of the solutions was adjusted so as to contain the total dose in a volume of 0.1 to 0.4 cc. Protoveratrine, veratridine, cevine, germine, and protoverine were dissolved in molecularly equivalent amounts of hydrochloric acid, buffered to approximately pH 7.0 with sodium bicarbonate, and injected as a solution of the hydrochloride in 0.9% sodium chloride. Jervine and rubijervine were dissolved in a small amount of glacial acetic acid, buffered to approximately pH 6 with sodium bicarbonate, and injected as the alkaline acetate.

The experiments on the isolated frog heart were performed between September and

November 1943 The hearts were taken from *Rana pipiens* and were either isolated according to Straub or perfused from the venous side using the technique described by Büllbring. Clark's solution was used. For details see (18).

Seventeen experiments on the denervated heart-lung preparation were performed. Defibrinated blood was used. The dogs weighing between 7 and 15 kgm. were anesthetized with nembutal<sup>\*</sup> 85 mgm. per kgm. For further details of the methods used see (19).

The innervated mammalian heart was used in seven experiments, two of these were heart-lung head preparations and in the other five perfusion of the head was established from a donor dog. Dogs weighing from 9 to 12 kgm. were used for the heart-lung preparation. The donor dogs for the head perfusion weighed between 17 and 21 kgm. The blood for both circuits was heparinized<sup>†</sup>. The head including the carotid sinus areas was connected with the heart-lung circuit by nervous pathways only, the efferent fibers being represented by the vagus and sympathetic nerves. The methods of preparation and recording have been described in detail in earlier communications (20-21). Nembutal was used as an anesthetic agent in six experiments, only one of the head perfusion experiments was made under chloralose, 0.09 grams per kgm.

Changes in arterial blood flow were studied in the femoral arteries of seven dogs using differential manometers adapted from the method of Fleisch (22) and attached to arterial cannulae as previously described (23).

The mechanism of vagal block in the dog was studied in six animals under nembutal anesthesia. Both vagi were sectioned, and stimulating electrodes attached to a Harvard Inductorium were fixed in position on the peripheral segment of the right vagus nerve. Stimulation was accomplished by means of rhythmic make and break shocks at a frequency of about 180 per minute timed by the oscillations of a metronome. Blood pressure was recorded from the left carotid artery with a mercury manometer and heart rate with an electrocardiograph. In four experiments injections of acetylcholine or acetyl-beta-methylcholine were made into the superior vena cava through a cannula in the right external jugular vein.

The study of the release of epinephrine from the suprarenal glands under the influence of protoveratrine was carried out in dogs and in cats. In two dogs blood was withdrawn from the suprarenal vein before and during the blood pressure rise resulting from a dose of protoveratrine. The blood was then tested for its epinephrine-like action on the isolated strip of the small intestine of the rabbit using the arrangement of Magnus and quantitated by comparison with the effect of known concentrations of epinephrine hydrochloride.

In four experiments on female non-pregnant cats the rise in blood pressure and the depressant effect on uterine activity served to indicate the release of epinephrine. The animals were anesthetized by the intraperitoneal administration of 35 to 40 mgm. of nembutal per kgm. body weight, eviscerated and given artificial respiration. The vagi were cut to avoid vagal reflex action. Injections of protoveratrine were made into the celiac artery. Blood pressure was recorded from one of the carotid arteries by means of a mercury manometer. The movements of a segment of a horn of the uterus were recorded by means of a Cushny myocardiograph. Single injections or continuous infusion of epinephrine hydrochloride served for comparison. All dosages and concentrations mentioned refer to the epinephrine base.

For the circulatory experiments protoveratrine was dissolved in N/10 hydrochloric acid 0.1 cc. for every 5 mgm. and the solution adjusted to the desired volume with 0.9% sodium chloride solution. As there is the possibility that hydrolysis of this alkaloid may occur readily on standing a fresh solution was prepared every time on the day of the experiment. Prior to the injection the pH of the solution was brought to about 7.0 by addition

<sup>\*</sup> The nembutal used in this work was generously supplied by Abbott Laboratories North Chicago, Illinois.

<sup>†</sup> The substance used in these experiments was Laqueamin which was generously supplied to us by Hoffmann-La Roche, Inc. Nutley, New Jersey.

of sodium bicarbonate Whenever a solution had to be injected into the arterial blood supply of the head the temperature of the solution was brought to 38°C and the total volume injected did not exceed 0.2 cc Unless otherwise stated, all dosages mentioned in this paper refer to the alkaloidal bases

**RESULTS** *I Comparative toxicity of the veratrum alkaloids* A comparison of the toxicity of a larger number of the veratrum alkaloids has not been possible so far because apparently no investigator had available pure substances in a sufficiently large quantity As the potency of the alkamines is rather low, a small laboratory animal like the mouse had to be chosen in our experiments to utilize best the relatively limited amounts of the alkamines at our disposal

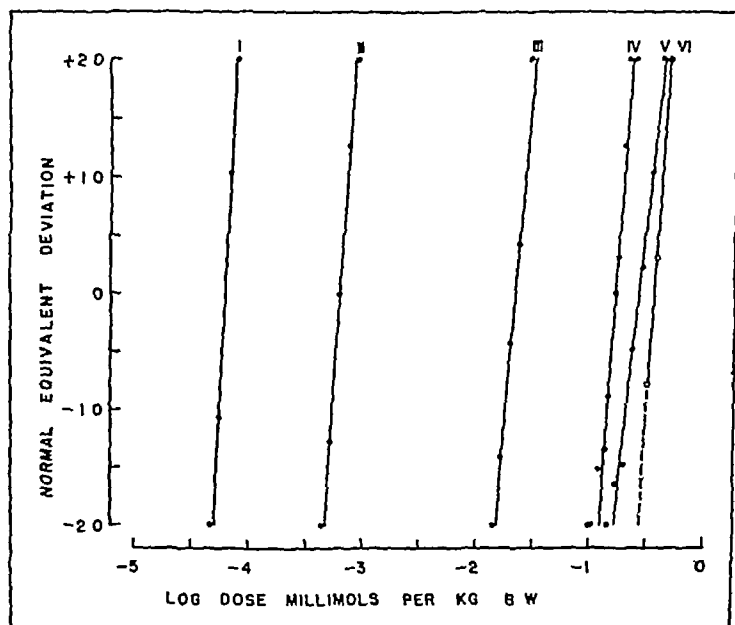


Fig 1 Mice

LD 50 For details see text I, protoveratrine, II, veratridine, III, jervine, IV with filled in circles, rubijervine, IV with open circles, cevine, V, germine, VI, protoverine

Further to do this and also to exclude differences due to absorption we selected the intravenous route

The results of our toxicity studies are illustrated in figure 1 As we used only six animals for each dose of each alkaloid, the deduced mortality was established from the observed mortality by the method of Behrens (24) From percentage mortality we determined the normal equivalent deviation as recommended by Gaddum (25) The average lethal dose, or LD 50, was then read from the straight line obtained by plotting the logarithm of the dose against the normal equivalent deviation These values are assembled in table 2

Apart from the marked quantitative differences there were also qualitative differences in the manifestations of poisoning caused by the various alkaloids as can be seen from the following observations

*Protoveratrine* There was a lag of about 15 minutes after the injection during which time no signs of poisoning could be recognized. Then followed a period of flaccid paralysis which was succeeded by convulsions in some cases in others by irregular respiration. Death occurred within 2 to 4 minutes.

*Veratridine* Respiration was depressed immediately after the injection. Death occurred within 1 to 4 minutes with signs of respiratory failure. In some cases a short period of convulsions preceded death.

*Cevine* Immediately after the injection tremor and convulsive movements occurred. Within a few minutes tremor became continuous the animals lay prone with legs and tail in extension. There was a great increase of reflex

TABLE 2  
*The toxicity of the veratrum alkaloids*  
(Mice intravenous injection)

SUBSTANCE	EMPIRICAL FORMULA	MOLE. WEIGHT	LD 50	LD50
			mgm./kgm.	microgram per kgm.
Protoveratrine	$C_{27}H_{47}O_5N$	751	0.043	0.064
Veratridine	$C_{26}H_{45}O_5N$	673	0.42	0.623
Jervine	$C_{27}H_{49}O_5N$	425	0.8	21.9
Rubijervine	$C_{27}H_{47}O_5N$	413	70.0	170.0
Cevine	$C_{27}H_{47}O_5N$	500	87.0	170.0
Germinine	$C_{27}H_{47}O_5N$	500	139.0	274.0
Protoverine	$C_{27}H_{47}O_5N$	525	194.0	387.0

excitability to touch and sound particularly to sharp high frequency noises like that produced by quickly aspirating air through the partly closed lips. When the poisoning followed this course death occurred as a rule within one-half to three hours. A few animals, however, died within 2 minutes after the injection. They had moderate convulsive movements and a slow and labored respiration. In some of the animals that escaped death the tremor lasted for several hours.

*Germinine* Almost immediately after the injection the animals became jumpy and had convulsive seizures. Within 1 to 3 minutes they were unable to stand they lay on their sides and had severe convulsions. Breathing was slow and irregular. Death generally occurred at this time. Animals escaping death regained the sitting position and occasionally had periods of tremor and jerking. Several hours elapsed before all toxic symptoms had subsided.

*Protoverine* About 30 seconds after injection the animals had brief fits of coarse tremor especially of the head, which bobbed up and down rapidly. As a rule death occurred within 5 minutes preceded by a short period of convulsive movements. Some animals died within 1 to 2 minutes. They showed severe jerking movements and death was preceded by a period of slow and

gasping respiration In those animals which recovered, fits of tremor were observed, they became less and less frequent and subsided within 1 to 2 hours

*Jervine* Thirty seconds after the injection great motor excitation—jumping and running—was noticed Repeated fits of convulsions were observed, and death occurred during a convulsive attack within a period of 10 minutes

*Rubijervine* The injection was followed immediately by either mild motor excitation or by a decrease in motor activity In some animals the outstanding symptoms were fits of convulsions or tremor, in others respiratory depression Death occurred within  $\frac{1}{2}$  to 2 minutes either in a convulsive attack or as a result of respiratory failure

*II Circulatory action of protoveratrine* *A Heart action* *Action on the isolated frog heart* Boehm (14) studied the effect of different concentrations of protoveratrine on the isolated frog heart He states that protoveratrine causes standstill of the ventricle in diastolic position and that the time necessary to produce the effect varies in proportion to the concentration, from 7 minutes for a concentration of  $1 \times 10^{-3}$  to 70 minutes for a concentration of  $1 \times 10^{-6}$

In our experiments, in the heart isolated by Straub's method, protoveratrine in concentrations of  $1 \times 10^{-3}$  to  $1 \times 10^{-4}$  caused ventricular standstill in the systolic position within 5 minutes (fig 2A) A concentration of  $4 \times 10^{-5}$  to  $1 \times 10^{-4}$  (fig 2B) produced an immediate "systolic effect" with frequent periods of contractions alternating with others in which the ventricle appeared as a whole "systolic," but close observation revealed fine "peristaltic" movements during which different parts of the ventricle contracted, alternately There was at this stage complete auriculo-ventricular dissociation Concentrations of  $1 \times 10^{-4}$  to  $2 \times 10^{-7}$  caused an increase in amplitude sometimes accompanied by a decrease in heart rate (fig 2c) This was usually followed by half-rhythm and after a period of approximately 2 hours by diminished contraction in a "half-systole" position of the ventricle The effect of protoveratrine on the isolated frog heart is reversible by washing, as was already noticed by Eden in his experiments using the Williams apparatus

The hypodynamic frog heart was used to study the therapeutic efficiency of protoveratrine The hearts were perfused from the venous side with a constant venous supply and the output measured The hypodynamic stage was induced by perfusing with Clark's solution containing only 40 to 50% of its normal content of calcium Perfusion with the drug dissolved in calcium-poor Clark's solution was started when the output was reduced to 50% or less of its normal value A concentration of  $1 \times 10^{-7}$  (four experiments) was found capable of increasing the output of the hypodynamic heart to nearly its normal value A concentration of  $1 \times 10^{-6}$  (four experiments) also increased the output but signs of toxic action, half-rhythm, and auriculo-ventricular dissociation appeared after 10 or 20 minutes of perfusion with the solution of protoveratrine

*Action on the denervated heart-lung preparation of the dog* Protoveratrine injected into the venous reservoir of the heart-lung preparation in doses of 0.005 to 0.02 mgm (concentration in the blood,  $1.17 \times 10^4$  to  $4.17 \times 10^4$ ) caused only slight or moderate improvement in the work capacity of the failing heart Right and left auricular pressures decreased slightly, and the total output of the

heart increased. The improvement developed more slowly than that caused by effective doses of veratridine and was never complete, that is auricular pressure and cardiac output were never restored to the control level. The trend of failure may be arrested for as long as 30 minutes after a dose of 0.01 mgm., an initial dose of 0.005 mgm. causes a moderate effect lasting about 15 minutes. If additional protoveratrine be administered when the effect of the first dose has reached a maximum no further improvement occurs and subsequent doses

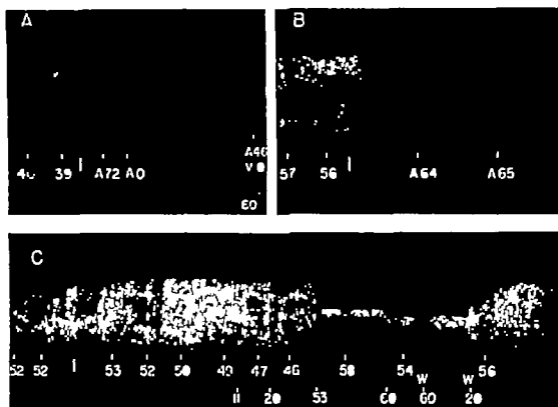


FIG. 2. ACTION OF PROTOVERATRINE UPON THE FROG HEART (STRAUB-CLARK'S SOLUTION)

Time in minutes. The figures above the time line indicate heart rate per minute. A = atrium, V = ventricle. The figures below the time line indicate in section A time interval in minutes between the two sections of the tracing; in C, period in minutes during which the drum was arrested. A 9/20/43. Temperature 24°C. Protoveratrine 1,000. The sinus was beating throughout. 5 washings within one hour brought the rate of the atrium back to 46 per minute, while the ventricle remained in systolic standstill. B 9/3/43. Temperature 24°C. Protoveratrine 100,000. Complete dissociation between atrium and ventricle was quickly established. Peristaltic movements of the ventricular muscle were noticeable in the periods of systolic standstill. Subsequent to the end of record B the periods of apparent systolic arrest became longer. C 9/20/43. Temperature 24°C. Protoveratrine 15,000,000. At W the heart was washed with normal Clark's solution.

may appear to hasten the failure. In one experiment (exp. 5) a total of six doses each of 0.005 mgm. was given within 40 minutes. Slight improvement followed only the first two doses. Ten minutes after the sixth dose of protoveratrine veratridine was injected in a dose of 0.15 mgm. followed by rapid and complete restoration of control levels of auricular pressures and cardiac output. The improvement although great was brief but recurred after an additional dose of veratridine.

Doses of 0.02 mgm. usually caused toxic manifestations (auricular asystole,

ventricular tachycardia, fibrillation) within 15 minutes, irregularities occurred almost immediately after the injection of larger doses, too soon to permit the development of a therapeutic effect in a failing heart

No heart rate changes were evident with sub-toxic doses. Coronary flow, as measured by introducing a Morawitz cannula into the coronary sinus, was not significantly altered

*Action on the innervated heart-lung preparation* Although protoveratrine in sub-toxic doses causes no significant heart rate changes in the denervated heart-lung preparation, bradycardia is one of the characteristic features of the action of this alkaloid in the intact animal. The mechanism of cardiac slowing was studied in the innervated heart-lung-head preparation and in head perfusion experiments. Heart rate was recorded in all cases with the electrocardiograph. In the heart-lung-head preparation protoveratrine was injected into the blood stream between the venous reservoir and the right atrium. The alkaloid was thus distributed in a total blood volume of about one liter, and reached not only the heart and lungs, but also the carotid regions and the central nervous system.

The minimal effective dose of protoveratrine in the heart-lung-head preparation was approximately 1 to 2 micrograms (order of concentration, when reaching the heart for the first time, 1:30 to 1:15 million), which, because of the restricted circulation, is considerably less than the effective cardiodecelerator dose in the intact animal. Unlike veratridine, which characteristically leads to an abrupt decrease in heart rate with a gradual return to normal level, protoveratrine caused a progressive decrease in heart rate for from 1 to 3 minutes, followed by a slow recovery which was often incomplete.

When small doses were given, the effect could be repeated several times, but eventually tachyphylaxis developed. When the vagi were cut during the period of bradycardia, heart rate increased at once, and cardiac slowing could no longer be induced by doses of 0.015–0.032 mgm of protoveratrine. Progressively increasing doses (to 0.03 mgm or more) eventually led to cardiac irregularities as in the ordinary denervated preparation.

These experiments point to the rôle of the vagus, but prove only that efferent vagal pathways are involved, information as to the reflex or central nature of the bradycardia is best gained from the preparations in which the cephalic and thoracic circulations are completely separated. In three such head perfusion experiments protoveratrine was injected in varying doses into the arterial blood supply to the head, or into the blood stream of the heart-lung preparation.

Bradycardia was produced by doses of 5 micrograms injected into the heart-lung circulation (fig 3A, part A, fig 3B, heart), in which case the alkaloid must have excited afferent endings in the heart and/or lungs, since such doses have no effect on the rate of the denervated heart, and access of the injected material to the centers was prevented. Doses of 10 to 30 micrograms, injected into the cephalic circulation, also resulted in cardiac slowing (fig 3A, part B, fig 3B, head). In the experiment in which chloralose anesthesia was used, no significant difference in the dose range or the character of the response was observed, moderate slowing was obtained with 3 micrograms in the heart circulation and

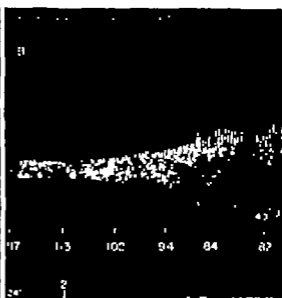
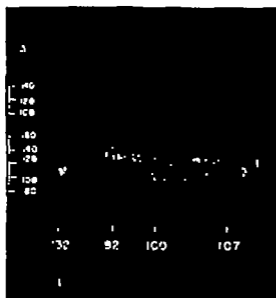


FIG 3a EXPERIMENT 4/26/44 REFLEX AND CENTRAL EFFECT OF PROTOVERATRINE UPON THE HEART RATE

Head perfusion experiment innervated heart-lung preparation Heart lung dog, female 100 kgm Donor dog female 18.2 kgm Nembutal anesthesia, 30 and 33 mgm per kgm respectively Heparin 3 mgm per kilo to both dogs and continuous infusion of heparin to donor dog at a rate of 1 mgm per minute Temperature of heart-lung preparation 38.5°C rectal temperature of donor dog, 30 C Tracings from top to bottom systemic output of heart in 100 cc (Weese stromuhr); time in 10-second intervals arterial blood pressure in the head circulation, scale at left in mm Hg, right auricular pressure of heart-lung preparation scale at right in mm. water signal line The horizontal row of figures indicates heart rate per minute of heart lung preparation

At 1 in A injection of 5 micrograms of protoveratrine into blood supply of heart-lung preparation At 2 in B injection of 10 micrograms of protoveratrine into arterial blood supply to the head of the recipient dog Between A and B there was an interval of 24 minutes

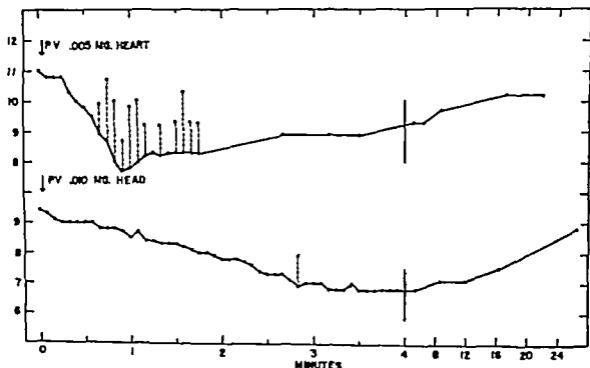


FIG 3b HEART RATE CHANGES OF THE EXPERIMENT FROM WHICH FIGURE 3a WAS TAKEN (Upper curve represents segment A lower curve segment B) Ordinate represents heart rate per 5 seconds The points connected by dashes with the solid line (auricular rate) of the upper curve represent actual ventricular rate during a period of ventricular extrasystoles

with 5 micrograms in the head. No attempt was made to determine the rôle of the carotid sinus areas in this response, in some experiments with veratridine (21) these receptors appeared to contribute to the overall effect.

It can be said, then, that protoveratrine, like veratridine, leads to a bradycardia, in part as the result of a reflex action originating in the thoracic viscera, and in part due to a central or cephalic action. In either case the efferent pathway lies chiefly in the vagus nerves.

*B. Action on the intact circulatory system of the dog.* Protoveratrine injected into the right atrium in doses of from 1 to 3 micrograms per kgm produced a

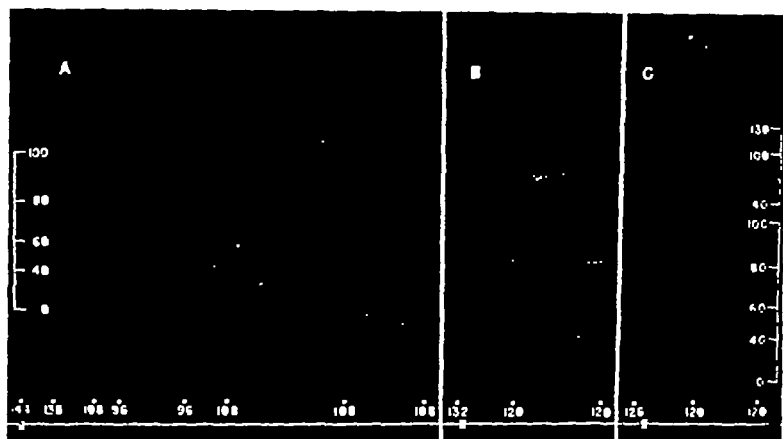


FIG 4 EXPERIMENT 6/11/43 DOG, 15.5 KGm, NEMBUTAL ANESTHESIA

Tracings top to bottom: respiration, recorded from side tube in tracheal cannula, carotid pressure scale at right in mm Hg, blood flow in left femoral artery (leg acutely denervated), scale at left in cc per min, blood flow in right femoral artery (nerve supply intact), scale at right in cc per min, time in 5-second intervals, signal line. The horizontal row of figures indicates heart rate per minute (counted for 5-second periods).

At signal in A, protoveratrine, 0.02 mgm, injected into superior vena cava. Between A and B, one additional dose of 0.02 mgm protoveratrine, time lapse of 50 minutes. At signal in B, protoveratrine 0.02 mgm. Between B and C, time lapse of 8 minutes. At signal in C, veratridine, 0.1 mgm.

fall of arterial pressure and a decrease in heart rate. The response was less abrupt than that following administration of comparable doses of veratridine. Arterial pressure began to decrease after a latency of 15 to 30 seconds, and reached a minimum in from 1 to 3 minutes. The gradual course of the pressure curve, as contrasted with that recorded for veratridine, appears to be due to the slower onset of bradycardia. The latency for the onset of cardiac slowing varied from 40 to 120 seconds, the minimum heart rate was reached in from 1 to 3 minutes. The depressor response lasted much longer than with veratridine. In the experiment of figure 4 (exp 2), arterial pressure dropped from 116 mm Hg to 68 about one minute after the injection of 0.02 mgm (1.25 micrograms per kgm) of protoveratrine, at the end of 35 minutes the mean pressure was relatively level at 85 mm Hg.

Vasodilatation contributes to the fall in pressure as with veratridine (fig 4). The blood flow in the innervated femoral artery in this experiment was increased by 400% after a dose of 0.02 mgm while mean arterial pressure was decreased by 40%.

In contrast to the effects of veratridine doses of protoveratrine which result in profound effects upon the circulation are without apparent respiratory action.

*Reflex nature of vasodilatation* Intra arterial injection of protoveratrine in doses approximating those effective intravenously and leading to local concen

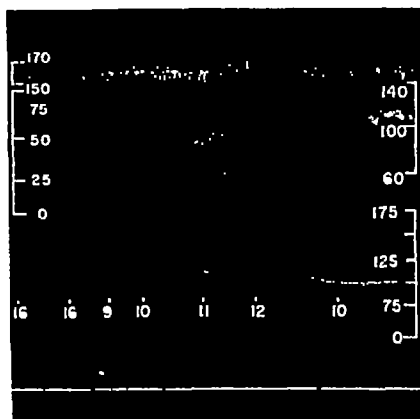


FIG 5 EXPERIMENT 3/3/44 DOG 18.5 KGm NEMBUTAL ANESTHESIA

Circulation of right leg (innervation intact) supplied by donor dog. Tracings from top to bottom: carotid pressure of donor dog (perfusion pressure of right femoral artery of recipient) scale at left in mm Hg; carotid pressure of recipient dog scale at right in mm Hg; blood flow in left femoral artery, scale at left in cc per min; blood flow in right (perfused) femoral artery scale at right in cc per min; time in 5-second intervals. At signal protoveratrine 0.076 mgm was injected into the superior vena cava of the recipient dog. The horizontal row of figures indicates heart rate in 5 seconds.

trations of 1:100,000 to 1:25,000 were without action on the femoral arterial flow suggesting that dilatation must be indirect. In two experiments perfusion of one lower extremity was established from a donor dog. Flow responses were studied in both perfused and intact femoral arteries. Protoveratrine injected intravenously into the recipient animal caused vasodilatation in both femoral arteries (fig 5); this response is evidently mediated via vasomotor fibers in the sciatic and femoral nerves.

*Tachyphylaxis* While veratridine may be injected repeatedly with little or no diminution in the depressor and vasodilator responses, protoveratrine leads

to marked tachyphylaxis. In the first experiment of this series it was observed that after the first effective dose of protoveratrine (0.032 mgm) had been given, a depressor or cardiodecelerator response could no longer be elicited, even with doses of 0.1 mgm. Veratridine was then injected in a dose of 0.1 mgm (4 or 5 times the usual minimal effective dose) and no effect on blood pressure, femoral flow, or heart rate occurred. Stimulation of the peripheral end of the right vagus with a strong tetanizing current from a Harvard inductorium was likewise without effect upon heart rate.

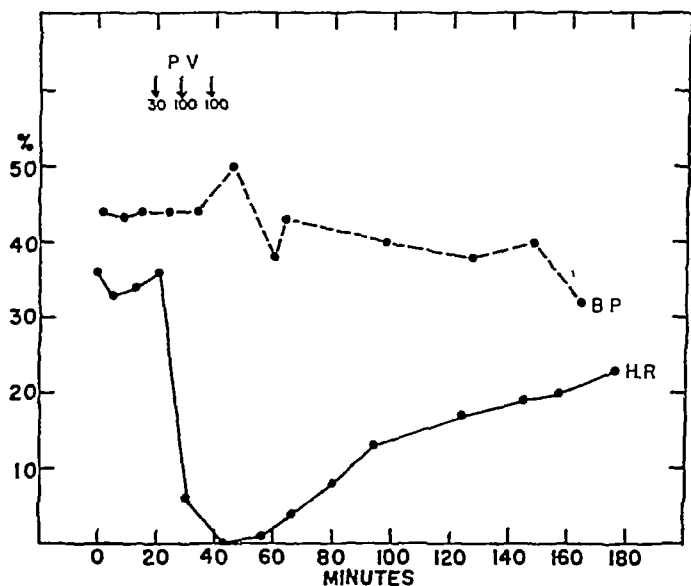


FIG 6 EXPERIMENT 2/1/44 DOG 18 KGM, NEMBUTAL ANESTHESIA

Both vagi cut, stimulation of right peripheral vagus. H.R. percentage decrease in heart rate during brief periods of vagal stimulation. B.P. percentage decrease in arterial pressure following repeated doses of 0.040 mgm acetylcholine chloride. At arrows, protoveratrine 0.03, 0.1, and 0.1 mgm intravenously.

In six dogs weighing from 16 to 22 kgm. The heart rate response to peripheral vagal stimulation was studied before and after injection of protoveratrine. The normal response to stimulation was established by several control determinations, after which protoveratrine was injected intravenously in single or divided doses totalling from 0.1 to 0.3 mgm. Within 1 or 2 minutes after the injection of such doses, the response to vagal stimulation was greatly decreased or abolished (fig. 6). Repeated determinations demonstrated a gradual recovery toward normal, which was still incomplete after 3 hours. This confirms the observations of Eden.

If this response were a true atropine-like action, the depressor action of choline esters should likewise be abolished. As shown in figure 6, the blood pressure

response to acetylcholine in repeated doses of 0.04 mgm (which failed to cause cardiac slowing) was not altered by protoveratrine. In another experiment the heart rate response to injections of acetyl-betamethyl choline, however, was reduced (fig 7). Examination of the electrocardiographic tracings reveals that while acetyl betamethyl choline administered by injection or acetylcholine liberated by vagal stimulation, are less effective in causing a decreased heart rate after administration of protoveratrine they still exert an action on the heart. The records suggest that during vagal stimulation under these condi

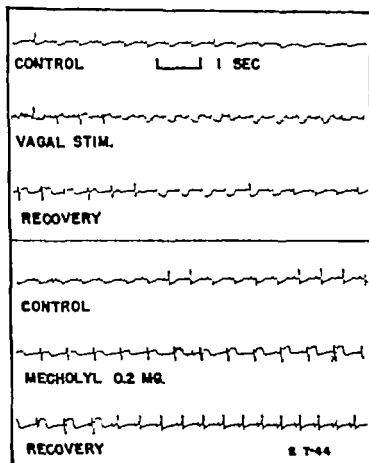


FIG 7 EXPERIMENT 2/7/44 DOG 16 KG. NEMBUTAL ANESTHESIA

Electrocardiographic tracings taken after 0.1 mgm. protoveratrine. Vagal stimulation causes no heart rate change but alters the ventricular complexes. Mecholyl causes little decrease in heart rate and also alters the pattern of the ventricular complex.

tions the normal pacemaker is depressed and an ectopic rhythm below the A V node is established (fig 7). Veratridine in doses of 0.1 to 0.2 mgm lacks this action. This effect of protoveratrine appears to be one exerted on the automatic properties of the myocardium rather than on either the vagus nerves or the humoral transmission mechanism in contrast to the action of atropine after the injection of which neither cardiac slowing nor development of an ectopic focus occur during vagal stimulation.

*C Blood pressure increase due to protoveratrine and the rôle of epinephrine release in the response. Experiments on the dog* After vagotomy or after doses

of protoveratrine which have "blocked" the vagi, doses of 0.1 to 1.0 mgm of the drug result in a hypertension and tachycardia similar to that resulting from somewhat larger doses of veratridine. The rôle of epinephrine in this response has been tested by several methods.

In two experiments on dogs in which femoral arterial flow was recorded, samples of blood were taken from the left adrenal vein before and after the injection of protoveratrine. The epinephrine content of the blood was assayed by means of the isolated rabbit gut. While control samples were without significant inhibitory action, the samples obtained during the height of the pressor and tachycardiac response contained epinephrine in concentrations of approximately 1:100,000. In order to rule out the effect of changes in adrenal blood

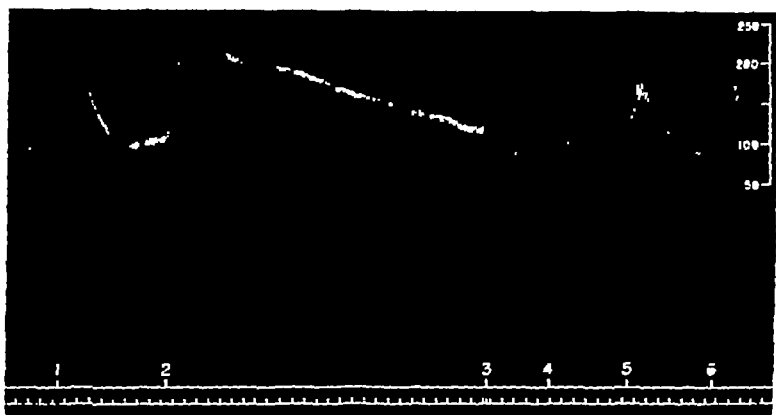


FIG. 8. EXPERIMENT 8/20/43. CAT 2.7 KGm, NEMBUTAL ANESTHESIA, EVISCERATED, VAGI CUT.

Tracings from top to bottom: carotid pressure, scale at right in mm Hg; uterine contractions recorded with a Cushny myocardiographic lever; signal line; time in minutes.

At 1, epinephrine, 0.01 mgm, injected into right external jugular vein. At 2, protoveratrine, 0.01 mgm, injected into the celiac artery. At 3, time lapse of 8 minutes, ligation of adrenals. At 4, protoveratrine, 0.01 mgm, injected into celiac artery. At 5, epinephrine, 0.01 mgm, and at 6, 0.02 mgm intravenously.

flow, this was recorded by means of a graduated pipette in one experiment. Blood flow was doubled during the pressor response, and epinephrine release was of the order of 10 micrograms per minute. As a further check, the area of the anterior surface of the spleen was measured in one experiment. Following a dose of 1.0 mgm of protoveratrine the splenic silhouette area decreased from 130 cm<sup>2</sup> to 60 cm<sup>2</sup>.

*Experiments on the cat.* Further evidence of the release of epinephrine from the adrenal glands has been obtained by injecting protoveratrine into the celiac artery of anesthetized, vagotomized, and eviscerated female cats in which the rise in blood pressure and the depressant effect on the non-pregnant uterus were used as indicators for the liberation of epinephrine. Figure 8 shows the results obtained in an experiment of this type. An injection of 0.01 mgm of proto-

veratrine into the celiac artery caused an increase in blood pressure and a depressant effect on uterine movements comparable in intensity to, but much longer-lasting (18 to 34 minutes in three experiments in which this dose was administered) than that caused by an injection of 0.01 mgm of epinephrine into the jugular vein. The effect disappears after ligation of the suprarenals. A continuous infusion of epinephrine was administered into the entrance of the right auricle by means of a long cannula inserted through the jugular vein in order to evaluate the amount of epinephrine released per unit of time by the adrenals into the circulation under the influence of protoveratrine. Two experiments of this type in which the effect of a dose of protoveratrine was compared with that of a continuous infusion of epinephrine showed that 0.01 mgm of protoveratrine injected into the celiac artery produced a sustained increase in blood pressure similar to that caused by a continuous infusion of approximately 0.45 cc. of a solution of epinephrine 1:100,000 or 4.5 micrograms, per minute.

**DISCUSSION** The toxicity studies in mice have shown that protoveratrine is the most toxic of the veratrum alkaloids. On a molar basis its toxicity is about 10 times that of veratridine and 6000 times that of its own alkaline protoverine. This is in general agreement with earlier studies on the toxicity of the veratrum alkaloids, as may be seen from tables 3 and 4. In table 3 the older data have been assembled, taken from investigations in which toxicity was not well defined and in which the number of animals per dose was limited or was not stated. Also in some cases it is doubtful whether the substances tested were pure. Table 4 contains data from more recent work in which the LD 50 was established.

That the naturally occurring ester-alkaloids of the veratrum group are so much more powerful pharmacological agents than their alkamines calls to mind *similar pharmacological facts for example, the difference between the solanaceous ester alkaloids atropine and scopolamine, and their respective alkamines, tropine and scopine, in regard to their influence upon impulse transmission to autonomic effectors or the difference in the pharmacological action of choline esters and choline*.

From the data of Heinz (in table 3) it is evident that the introduction of an acetyl group into the molecule of cevadine decreases toxicity noticeably in frogs, as well as in rabbits but not to the same extent as the introduction of a benzoyl group. Dibenzoyl cevine with the tiglic acid also replaced by a benzoyl group has lost still more of its potency. The experiments of Haas (in table 3 and 4) on rats with subcutaneous injection of germerine, protoveratridine and germine show clearly that the removal of the methylethyl glycolic acid from germerine reduces the toxicity more than 250 times and protoveratridine the methylethyl acetic acid ester of germine, is but little more toxic than the alkaline germine itself. Apart from and independent of the genuine toxicity of the alkamines, the toxicity of the ester-alkaloids obviously is dependent on the number of acyl groups in the molecule as well as upon the nature and probably also the position of the individual acyl group.

Only jervine among the known alkamines investigated by us comes closer in

TABLE 3

ANIMAL SPECIES	WAY OF ADMINISTRATION	SUBSTANCE	LETHAL DOSE*		AUTHOR
			Mgm./animal	Mgm./kgm.†	
<i>Rana</i> (?)	Subcut	Protoveratrine	0.2	6.0	Eden (13)
<i>Rana</i> (?)	Subcut	Cevadine	0.05	1.5	Heinz (26)
<i>Rana</i> (?)	Subcut	Cevadine (sulfate?)		7.0	Pilcher (27)
<i>Rana escul</i> and <i>temp</i>	Subcut	Cevadine	0.5-1.0	15.0-30.0	Lissauer (28)
<i>Rana</i> (?)	Subcut	Acetyl cevadine hydrochloride	1.0	30.0	Heinz (26)
<i>Rana</i> (?)	Subcut	Benzoyl cevadine	>10.0	>300.0	Heinz (26)
<i>Rana</i> (?)	Subcut	Dibenzoyl cevine acetate	20.0	600.0	Heinz (26)
<i>Rana escul</i>	Subcut	Protoveratridine		>750.0	Haas (15)
<i>Rana escul</i>	Subcut	Germerine		250.0-500.0	Haas (15)
Guinea pig	Subcut	Cevadine	0.5		Heinz (26)
Rabbit	Subcut	Protoveratrine		0.11	Eden (13)
Rabbit	Subcut	Cevadine	1.0	0.5	Heinz (26)
Rabbit	Subcut	Cevadine	2.7 (-4.2)	1.3	Lissauer (28)
Rabbit	Subcut	Acetyl cevadine hydrochloride	50.0	25.0	Heinz (26)
Rabbit	Subcut	Dibenzoyl cevine acetate	>100.0†	>50.0	Heinz (26)
Rat	Subcut	Protoveratridine		>1000.0	Haas (15)
Rat	Subcut	Germerine		2000.0	Haas (15)

\* Unless otherwise mentioned the dosages refer to the base and not to the salt

† The boldface figures for lethal dose in mgm./kgm. were those actually reported. The others were calculated from the reported values for the whole animal to get an approximate dose per kgm. for the frog by multiplying by 30, assuming an average weight of 33 grams per frog, for the rabbit by dividing by 2, assuming an average weight of 2 kgm. per rabbit.

‡ This dose caused only slight depression of the central nervous system.

TABLE 4

ANIMAL	WAY OF ADMINISTRATION	SUBSTANCE	MOL. WEIGHT	LD 50		AUTHOR
				mgm./kgm.	micro-mols per kgm.	
<i>Rana esculenta</i>	Subcut	Protoveratrine	751	4.5	6.0	Haas (15)
<i>Rana esculenta</i>	Subcut	Germerine	679	9.0	13.2	Haas (15)
<i>Rana temporaria</i>	Subcut	Protoveratrine	751	13.5	17.9	Haas (15)
<i>Rana temporaria</i>	Subcut	Germerine	679	20.0	29.4	Haas (15)
Rat	Oral	Protoveratrine	751	5.0	6.7	Haas (15)
Rat	Oral	Germerine	679	30.0	44.1	Haas (15)
Rat	Subcut	Protoveratrine	751	0.6	0.8	Haas (15)
Rat	Subcut	Germerine	679	3.7	5.4	Haas (15)
Rat	Intrapert	Veratridine	673	3.5	5.2	Mendez and Montes (18)
Rat	Intrapert	Cevine	509	67.0	131.0	Mendez and Montes (18)

toxicity to the ester-alkaloids. The reason is unknown. It differs chemically from the other alkalamines (which are tertiary amines) in that it is a secondary amine. The toxic doses of rubijervine, cevine, gerrmine and protoveratrine are in the ratio of 2, 2, 3, 4, but no explanation is available at present for this relation. In view of the fact that cevine and gerrmine are isomers, it is of interest to be able to record definite quantitative as well as qualitative differences between these two alkalamines.

Protoveratrine is a more potent cardiac poison than veratridine, the former is toxic to the dog heart in doses as small as 0.015 mgm (concentration in the blood, 1:55,000,000) while the latter causes irregularities in doses of 0.65 mgm (concentration of about 1:1,000,000). It is very probable that the difference in cardiac toxicity is largely responsible for the difference between protoveratrine and veratridine in toxicity to the whole animal.

Protoveratrine is much less effective in producing a positive inotropic (therapeutic) effect on the heart. In two experiments veratridine caused great

TABLE 5

	VERATRIDINE (v)	PROTOVERATRINE (p)	v/p
I. H.L.P. minimum therapeutic dose	0.05 mgm.	0.005 mgm.	10
II. H.L.P., minimum toxic dose	0.65 mgm.	0.015 mgm.	43
III. Average depressor dose (dog)	0.03 mgm.	0.03 mgm.	1
IV. Average pressor dose (dog)	0.50 mgm.	0.10 mgm.	5
V. Mouse LD 50 micromols per kgm.	0.628	0.064	10
Ratio of II/I	13	3	
Ratio of II/III	22	0.5	

improvement in the failing heart after repeated small doses of protoveratrine had not prevented progressive failure. Although the minimum effective dose of protoveratrine, about 0.005 mgm, is only  $\frac{1}{13}$  of that of veratridine, the degree of improvement attainable is much less, and the margin between therapeutic and toxic doses is narrower. The ratio of toxic to minimum therapeutic dose for veratridine is about 13, and for protoveratrine not more than 3 (table 5).

Comparing the doses which are toxic to the heart with those that are effective in reducing the blood pressure of the intact animal, it is evident that effective depressor doses of protoveratrine lie in the range likely to cause cardiac irregularities. Electrocardiographic evidence confirms this, doses of 0.1 mgm frequently cause ectopic rhythms in the intact dog. Veratridine, which effectively reduces blood pressure in the same dosage range as protoveratrine, is obviously much safer in this regard; the effective depressor dose in the intact dog, 0.02 to 0.03 mgm, is only  $\frac{1}{3}$  to  $\frac{1}{2}$  the dose which may cause irregularities when confined to the small blood and tissue mass of the heart-lung preparation. These data are summarized in table 5.

The results of our experiments on the isolated frog heart do not confirm the

observations of Boehm, who did not see systolic standstill when using the same technique, while they agree with the work of Eden, who saw systolic standstill of the heart in the intact frog. However, he does not clearly state whether systolic standstill occurred in his experiments with the isolated frog heart using the Williams apparatus.

In general the effect of protoveratrine upon blood pressure and upon the response to electrical stimulation of the peripheral end of the severed vagus is in good agreement with and confirms Eden's findings. That Eden did not observe decrease in blood pressure without subsequent increase above the level before the injection most probably is due largely to his using relatively large initial doses, as he calls 0.05-0.1 mgm per animal "very small doses." This is also borne out by his observations on the marked tachyphylaxis as a result of which a second dose no longer caused a decrease in pressure at all. It is possible that his technique of immobilizing the experimental animals with ether and curare also contributed to the fact that he observed a secondary blood pressure increase with all his initial doses of protoveratrine.

Our explanation of the apparent vagal block produced by protoveratrine is in accord with the observations of Rothberger and Winterberg (29). Their electrocardiographic studies uncovered the fact that after poisoning with muscarine, physostigmine, digitalis, strychnine, and epinephrine, as well as during high intracardiac pressure, the response to faradic stimulation of the vagus is concealed by the idioventricular rhythm.

Although the action of protoveratrine in causing an apparent vagal block may explain the development of tachyphylaxis in the heart rate response to the drug, it does not explain the tachyphylaxis of the depressor or vasodilator responses. Protoveratrine must lead to a block either in the afferent pathways or in the centers or efferent routes involved in the mediation of vasodilatation. Such an explanation would be in accord with earlier observations of block produced in frog motor nerve by protoveratrine solutions (14).

Like veratridine, protoveratrine causes a vasodilatation in the femoral vascular bed by a reflex mechanism, and local effects of the drug do not appear to play any rôle in this action. Of experiments on isolated organs only studies on the perfused hind legs of the frog were made by Haas, who found in two out of three experiments a vasoconstriction if 0.5 cc of 1:100,000 of protoveratrine-Poethke was injected into the perfusion fluid.

It is not likely that vasoconstriction due to a direct action of protoveratrine upon the arterial resistance plays any rôle in the blood pressure increase after vagotomy, as concentrations of 1:15,000 reached in intra-arterial injections into the femoral arterial circuit proved ineffective and the actual concentrations of protoveratrine reached by intravenous effective doses are far less. We have shown that release of epinephrine from the suprarenal glands plays a part in the blood pressure increase.

#### SUMMARY

In acute toxicity experiments in mice with intravenous administration, protoveratrine, a triacyl ester of protoverine, was found to be ten times more toxic than

veratridine a monoacyl ester of cevine. The alkalamines jervine, rubijervine, cevine, germine, and protoverine were very much less toxic than the ester alkaloids. Conspicuous qualitative differences exist between the alkaloids and the alkalamines as groups as well as between the individual members of the two groups.

In conformity with its sterol nature and chemical relation to the cardiac glycosides, protoveratrine causes a systolic standstill in the isolated frog heart and positive inotropic (therapeutic) effect in the hypodynamic frog heart, as well as in the isolated failing mammalian heart. The minimal effective therapeutic dose is somewhat smaller than that of veratridine. The minimal toxic dose leading to irregularities of the heart rate is very much smaller than that of veratridine. Effective inotropic doses of protoveratrine not leading to irregularities of rate do not decrease the heart rate of the denervated heart, this is contrary to what was observed with veratridine. In the innervated heart, however, decrease in heart rate is produced which is partly due to reflex action and partly due to central action as was observed with veratridine.

In the intact circulation of the dog the outstanding effect of small dosages (0.5 to 2.5 micrograms per kgm.) is a blood pressure decrease which is due in part to a heart rate decrease and in part to a decrease in arterial resistance.

The vasodilatation as studied in the femoral artery is reflex in nature. Intrarterial injections of dosages leading to much higher arterial concentrations than would ever occur with intravenous administration having no effect.

As was observed with veratridine, large dosages of protoveratrine cause release of epinephrine from the suprarenal gland, which is an important element in the vasopressor effect.

Tachyphylaxis to the cardiodecelerator effect as well as to the depressor effect develops much more readily with protoveratrine than with veratridine.

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# CLINICAL STUDIES ON DIGITOXIN (DIGITALINE NATIVELLE)

## WITH FURTHER OBSERVATIONS ON ITS USE IN THE SINGLE AVERAGE FULL DOSE METHOD OF DIGITALIZATION

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In several reports (1, 2, 3, 4) from our laboratory and clinics during the past few years evidence was presented indicating that digitoxin (Digitaline Nativelle) possesses properties which place it first in the choice of digitalis materials for general therapeutic use. It is rapidly absorbed from the gastro-intestinal tract so that in animals effects are pronounced in as short a period as 15 minutes after a large oral dose (5). Its absorption is virtually complete so that for a particular effect it seldom requires more of the drug by mouth than by vein. There are very few drugs which are so well absorbed that the intravenous and oral doses are the same. The potency of digitoxin is so high that it requires a smaller amount of the drug to produce the full therapeutic effects by oral administration than in the case of any other digitalis material in common use at the present time. The doses necessary for full therapeutic effects are too small to produce gastro-intestinal irritation with the result that nausea and vomiting from local action seldom occur. The margin of safety between the therapeutic and the toxic dose is the same as in the case of digitalis (1).

The foregoing properties seemed admirably suited for a system of digitalization using a very large dose at one time instead of the smaller fractions as in the methods by which digitalis is commonly given, which require a period of from 24 to 48 hours for satisfactory digitalization. We described such a system with the use of Digitaline Nativelle two years ago (6). Several problems remained to be explored. There were also several questions which arose during its further use in our clinics as well as in the application of the method by others. The purpose of the present report is to give an account of more extended experience in the use of this material and to describe the results of experiments designed to throw additional light on the behavior of digitoxin, and on problems of dosage and methods of application. It may also be noted that whereas the previous observations were all made with a preparation of digitoxin produced in France, many of the present experiments were performed with a digitoxin material prepared in the United States.

**Drug.** The term digitoxin will be applied to the material used in this study. This name is not entirely satisfactory. The problems involved in the nomenclature were discussed in a previous report (1). There is some doubt of the chemical identity of the material with that which Schmiedeberg named digi-

toxin in 1875, although there appears to be no significant pharmacological difference. The preparation available for practical use is a chloroform soluble active principle isolated from *digitalis purpurea* containing a negligible amount of impurity. The material prepared in the Laboratoire Nativelle of Paris has a potency by the official cat method (USP XII) of 0.42 mg per kg (1). We tested several specimens made in the United States in the laboratory of Wyeth Inc and these had potencies of 0.47 mg (#D63), 0.50 mg (#30-455), 0.32 mg (#114), and 0.36 mg (#115). The latter two seemed to be the purest preparations and these were used in a large proportion of the present experiments. They were available to us in the form of the crystals and tablets. The source of the material will be designated in this paper by the letter "N"

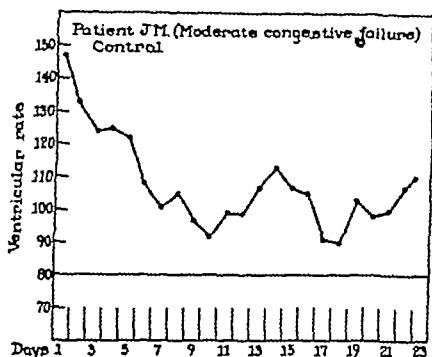


FIG 1 EFFECT OF BED REST ON THE VENTRICULAR RATE IN PATIENT WITH AURICULAR FIBRILLATION

Each point on the curve represents an average of three or more one-minute counts

for the product obtained from the Laboratoire Nativelle of Paris and the letter "W" for that from Wyeth Inc of Philadelphia

**OBSERVATIONS IN PATIENTS** In one of the methods used in this study the course of drug action was charted in terms of changes of the ventricular rate in patients with auricular fibrillation. It is well known that, although there are some exceptions, the decline of the ventricular rate is a good index of the therapeutic action of digitalis and runs fairly parallel with improvement in heart failure in patients with auricular fibrillation. The changes in rate are best suited to graphic representation. The patients were put to bed and the ventricular rate was counted 3 or more times daily during a control period. When the rate was stabilized, a dose of the drug was given and the rate was counted at intervals of an hour or less during the day. In the subsequent days of elimination of the drug, the rates were counted as in the control period. The importance of the control period before the drug was given is shown in figure 1. It frequently occurred that during the control period the ventricular rate declined to normal levels and simultaneously the symptoms of failure subsided as the result of rest in bed alone. Such patients were eliminated from the study

In previous reports (2, 3) it was shown by the behavior of single patients that equal effects are produced by a dose of digitoxin whether it is given intravenously or orally. In figure 2 this observation was established by the average of the results obtained in several patients with auricular fibrillation. After the control period each patient received a single dose of 1.25 mg. of digitoxin either orally or by intravenous injection. The record of each patient represented the one-minute counts made at frequent intervals during the day of the dose and the average of the 3 or more counts made during the control period and the days following the dose. The curves of figure 2 represent an average of several courses, 13 courses in 9 patients for the oral curve, and 20 courses in 14 patients for the intravenous curve. Four of the patients are represented in both curves. The results show that although the effects develop more quickly in the case of

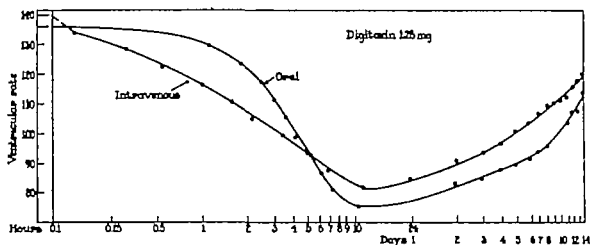


FIG 2 COMPARISON OF THE EFFECTS OF DIGITOXIN (N) 1.25 Mg. GIVEN ORALLY AND BY INTRAVENOUS INJECTION IN PATIENTS WITH AURICULAR FIBRILLATION

Time following the dose is spaced logarithmically. Note that while the intravenous dose produces effects more rapidly the total effect is no greater than after the same dose given orally.

the intravenous injection the total effect is substantially the same in the case of the oral doses: an average decline of 61 beats (oral) and 58 beats (intravenous) per minute. The absorption of digitoxin from the gastro-intestinal tract of patients is therefore substantially complete.

The foregoing results were obtained with the digitoxin (Digitalme Nativelle) prepared in France. Figures 3 and 4 show the behavior of the Wyeth materials in each of two patients under similar conditions. The results show that the patient is well digitalized by a single oral dose of from 1 to 1.5 mg. in a period of from 3 to 6 hours.

Reference has already been made to our previous study (6) in which digitalization with a single large dose of digitoxin was recommended for routine use. It was to replace the several fractions given at intervals of six to eight hours as in the usual plans for inducing digitalization with the leaf or the tincture and in this way to shorten the period required for inducing advanced digitalis effects. It is self-evident, however, that no single dose is to be found which

will produce exactly the same degree of digitalization in all patients. The amount required for optimum effects differs from patient to patient. The most tolerant individual may require more than twice as much as the most susceptible

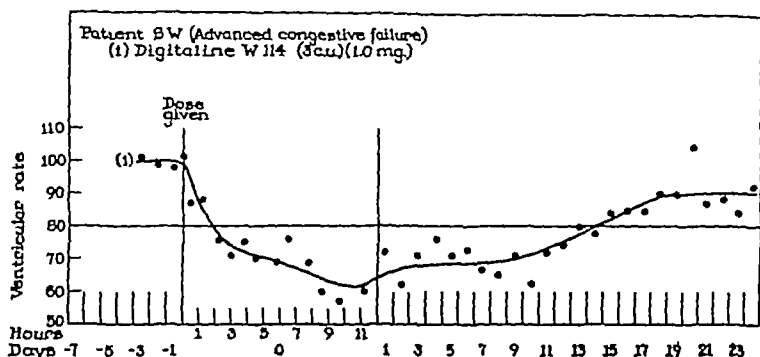


FIG 3 RAPID DIGITALIZATION WITH SINGLE DOSE OF DIGITOXIN (W) IN PATIENT WITH AURICULAR FIBRILLATION

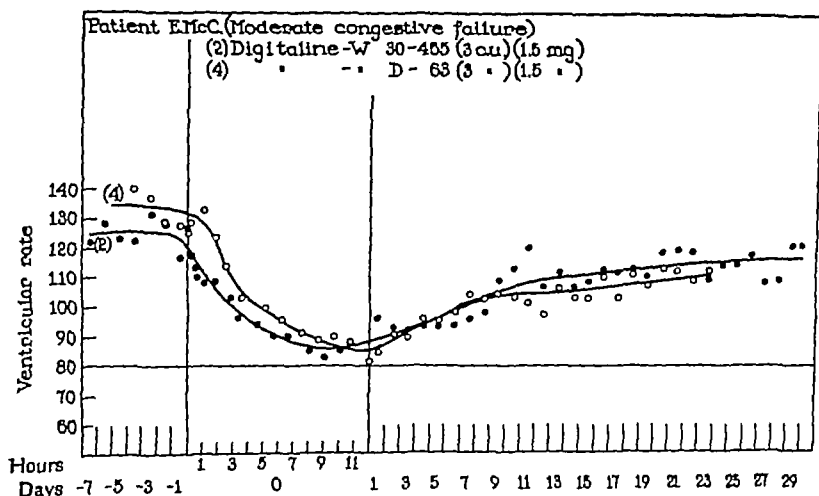


FIG 4 RAPID DIGITALIZATION WITH SINGLE DOSE OF DIGITOXIN (W) IN PATIENT WITH AURICULAR FIBRILLATION

Although these samples contained more impurities than the one in figure 3 the effects developed equally rapidly

person, even though tests on large populations of animals show that digitalis is among the drugs with relatively uniform action (7). That disease introduces factors which widen the range of individual variation in the required dose is well known. The more severe grades of heart failure are likely to require larger

doses (8) In auricular fibrillation without heart failure and in those with Graves disease larger doses may be necessary for the control of the ventricular rate (9) The size of a single full dose for routine use needs to be defined in relation to two factors, the incidence of satisfactory digitalization and the incidence of toxic effects when the dose is given to large numbers of patients The latter is the more important and it was in relation to the factor to toxicity that the 1.25\* mg dose of digitoxin was established as the most satisfactory for routine single dose digitalization The incidence of poisoning with this dose is negligible Up to the present time we have studied the effects of this dose in 512 patients. There were no signs of toxicity other than minor gastrointestinal symptoms in 2.8% of the patients This group of minor toxic reactions breaks down into mild nausea in 1 per cent (due to local action because it appeared in less than 2 hours), and nausea or vomiting occurring later (due in all probability to a systemic action) in 1.8 per cent of the patients The safety of the intensive method of digitalization with 1.25 mg of digitoxin given at one time seems therefore to be established

Many patients can tolerate and some require larger doses, but materially larger doses at one time are not appropriate for routine purposes This was determined in a series of 98 patients each of whom received a single dose of 2.0 mg of digitoxin In this group 32 per cent developed minor toxic effects, gastro-intestinal symptoms (21 per cent vomited in an average of 6 hours and 11 per cent had only nausea beginning in a few instances as early as 15 minutes after the dose) Most of these cases undoubtedly represent systemic toxicity

As to the therapeutic efficacy of the 1.25 mg dose of digitoxin, our experience shows that it carries the process of digitalization fairly far along in most patients with auricular fibrillation and moderate grades of heart failure What this dose represents in terms of digitalis leaf was established in two ways By one method, the effectiveness of digitoxin by oral administration in man was compared with that of digitalis leaf in the human method of assay described in 1942 (10) The digitoxin was tested against the U.S.P. Digitalis Reference Standard 1942 in six calibrated subjects with regular sinus rhythm using the changes in the T waves of the electrocardiogram as the basis of comparison. The results of this assay are summarized in table 1 By this method it was found that 1 mg of digitoxin exerts the effect of approximately 1 gm of the current U.S.P. digitalis by oral administration in man

By the second method the potency of digitoxin was compared with that of digitalis in terms of the reduction of the ventricular rate in patients with auricular fibrillation The results of these comparisons are presented in figure 5 The experiments were carried out in the manner described above for figure 2 The curve for digitalis represents the average of 24 courses of digitalization in 9 patients with 8 specimens of digitalis (leaf and tincture) The average dose was 16.3 cat units<sup>1</sup> given at one time the equivalent of 1.23 gm U.S.P.

In many of the recent observations the dose actually used was 1.2 mg which was found to be clinically indistinguishable from the 1.25 mg dose

<sup>1</sup> Fatal dose per kg when injected by the method described in the U.S.P. XII

"Digitalis Reference Standard 1942" (11) The second curve represents the average of 13 courses of digitalization in 9 patients with 1.25 mg digitoxin. Four of the patients are represented in both curves. The average ventricular rate before the drug was 132 in the case of digitalis and 136 in the case of digitoxin. The average fall of the rate as the result of the dose was 58 beats a min-

TABLE 1  
*Human assay of digitoxin*

NAME	EQUALLY EFFECTIVE AMOUNTS OF		POTENCY OF 1 MG DIGITOXIN IN MG OF U.S.P. DIG. REF. STAND. 1942
	Dig. ref. stand. 1942	Digitoxin (N)	
	mg	mg	
He Br	825	0.9	916.7
Ne Ro	825	0.8	1031.3
Vi Av	675	0.8	843.7
Fa Bo	816	0.9	906.7
Na Go	550	0.4	1375.0
Et Le	500	0.6	833.3
Average			984.5

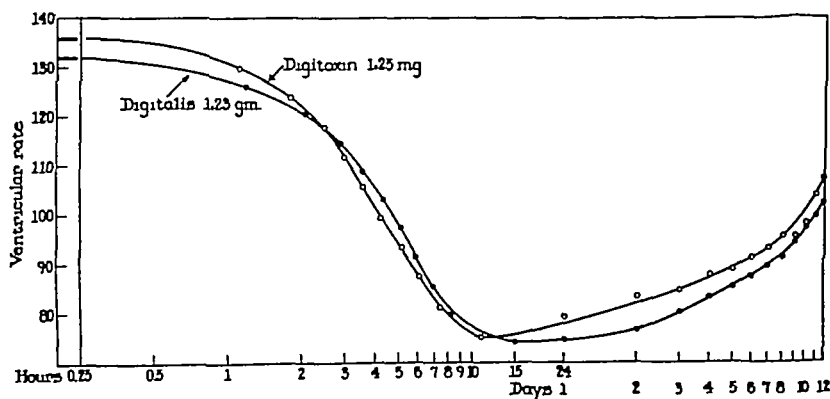


FIG 5 COMPARISON OF DIGITOXIN (N) AND DIGITALIS BY ORAL ADMINISTRATION IN PATIENTS WITH AURICULAR FIBRILLATION

Time following the dose is spaced logarithmically. Note that the 1.25 mg of digitoxin produces the same effect as approximately 1.25 gm of digitalis by oral administration in man.

ute in the case of digitalis and 61 in the case of digitoxin. The results, using the therapeutic action on rate, therefore, confirm the relative potency of the two shown by the method using the electrocardiogram, namely a ratio of approximately 1 to 1,000.

It may be noted that the two curves are practically identical throughout their course indicating that therapeutically comparable doses of digitoxin and digitalis are similar with respect to the speed of absorption and elimination. They

are sufficiently alike to suggest that the action of oral digitalis may be due to its most absorbable fraction, namely its content of digitoxin.

Since digitoxin is approximately 1 000 times as potent by oral administration in man as the current U.S.P. digitalis the single dose of 1.25 mg. of digitoxin produces the effects which may be expected from a dose of approximately 1.25 gm. of digitalis. Such a dose of digitalis is not much smaller than the total amount (about 1.5 gm.) of digitalis recommended in the various fractional dose methods (12 13 14). However it has already been shown in our previous study (6) and by others (15) that such a dose in the form of digitalis or its tincture cannot be given at one time since it causes gastro-intestinal symptoms in about one-fifth of the cases, in the majority due to a local irritant action.

It was shown several years ago (16) that the cumulation of digitalis is a self limiting process and that it is possible to induce full digitalization by the daily

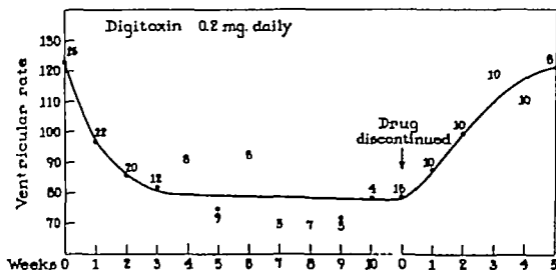


FIG. 6 CURVE OF DIGITOXIN (W) CUMULATION

Average of 26 courses in 17 ambulatory patients with auricular fibrillation. Figures at points indicate number of cases represented.

repetition of a small dose which may then be continued as the daily maintenance dose. This has been shown to apply to Digitaline Nativelle (1). Figure 6 shows the characteristics of this phenomenon for digitoxin in terms of the average of a group of patients. The curve is based on the results of observations in 17 adult ambulant patients with auricular fibrillation and heart failure in attendance at the cardiac clinics. All the common etiological varieties of heart disease are represented. The patients rested about an hour before the ventricular rate was counted at the apex. They received a supply of tablets of 0.1 mg. each and were instructed to take 2 tablets every evening. They were usually re-examined at intervals of a week. The curve represents the average of the results in 26 courses of treatment. It may be noted that by the daily administration of 0.2 mg. of digitoxin the ventricular rate in patients with auricular fibrillation declined gradually from 124 to 80 in about 3 weeks and continued to maintain that level during the subsequent period of 10 weeks in

which the same daily dose was taken. When the drug was discontinued the rate began to mount reaching the control level in a period of about 3 weeks.

Observations on the daily maintenance dose were made in these ambulant cases. Of 19 patients receiving a daily dose of 0.2 mg, three developed nausea and/or vomiting (16 per cent). One that received 0.4 mg daily developed vomiting and of 8 that received 0.3 mg daily three developed vomiting (37.5 per cent). The dose of 0.2 mg seems to be the most satisfactory daily maintenance dose for routine use.

It is our practice in the use of digitoxin in the undigitalized patient to give 1.2 mg at one time and follow this by a daily maintenance dose of 0.2 mg. The small daily dose not only serves to maintain the effects but also to complete the full digitalization in those cases in which the initial dose by itself proves inadequate. Very susceptible and very tolerant patients, and those under the partial influence of digitalis require corresponding adjustments in dosage.

#### SUMMARY AND CONCLUSIONS

1 This report deals with experience in the use of digitoxin in more than 1000 patients with heart disease, of which 722 were employed in experiments designed to throw further light on the behavior of digitoxin in man.

2 A dose of digitoxin is equally as effective by oral as by intravenous administration in man. This indicates complete absorption.

3 Digitoxin is approximately 1,000 times as potent as current U.S.P. digitalis leaf by oral administration in man, 1 mg of digitoxin produces the effects of approximately 1 gm of digitalis.

4 Evidence is presented which shows that the human method for the assay of digitalis materials, using the changes in T-wave of the electrocardiogram as a measure, tests their therapeutic activity.

5 The characteristics of the curve of digitoxin cumulation and elimination are similar to those of digitalis.

6 The most favorable maintenance dose of digitoxin for routine use is 0.2 mg daily.

7 A dose of 1.2 mg of digitoxin may be given at one time with complete safety. It produces the therapeutic effects of approximately 1.2 gm of digitalis. The incidence of minor toxic effects is negligible with this dose, whereas its equivalent in the form of digitalis cannot be given at one time because of the frequency of gastro-intestinal irritation.

8 The present study confirms our previous reports to the effect that digitoxin provides a safe means for rapid and intensive digitalization starting with a large single dose. This dose, 1.2 mg, suffices to produce full digitalization in the average patient with a moderate degree of heart failure. This method is recommended for routine use to replace the much slower techniques of digitalization with the smaller fractional dose methods required in the case of digitalis leaf or its tincture.

These studies were supported in part by the Digitalis Fund of Cornell University Medical College which includes contributions from Sharp and Dohme Wyeth Inc., Sandoz Chemical Works, Warner Institute for Therapeutic Research, Laboratoire Nativelle, Lederle Laboratories, Ciba Pharmaceutical Products, and Eli Lilly and Company

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# ISOPROPYL ALCOHOL RATE OF DISAPPEARANCE FROM THE BLOOD STREAM OF DOGS AFTER INTRAVENOUS AND ORAL ADMINISTRATION<sup>1</sup>

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The increasing use of isopropyl alcohol in the form of rubbing alcohol has created certain opportunities for human consumption. The acute and chronic effects of the alcohol in experimental animals has been described by Lehman and Chase (1), but comparatively little is known regarding its metabolism. Pohl (2) was among the first to show that acetone is a metabolic by-product. The rate of metabolism has never been quantitated although Morris and Lightbody (3) suggest that this is a slow process. Because of the negative character of the known facts concerning the metabolism of isopropyl alcohol it was deemed desirable to investigate this phase of the problem as a part of a systematic pharmacologic study. Certain observations have been made on the rate of disappearance of isopropyl alcohol from the blood stream of dogs following intravenous and oral administration and the present paper presents the results of such a study.

**PROCEDURE** Isopropyl alcohol was administered intravenously and orally to normal fasting dogs. For the intravenous injections the alcohol was diluted to a concentration of 20 per cent in 0.9 per cent sodium chloride solution and given by gravity into a leg vein. The rate of flow was approximately 0.25 cc per kilogram per minute. The total dose ranged from  $\frac{1}{4}$  to  $\frac{3}{4}$  of the intravenous fatal dose previously established under similar conditions (1) and represented 0.64 cc to 3.84 cc per kilogram of absolute alcohol. For the gastric administrations a 25 per cent dilution of the alcohol in warm tap water was employed in doses ranging from  $\frac{1}{4}$  to  $\frac{1}{2}$  of the gastric fatal dose (1) and represented a total quantity of 0.93 cc to 3.75 cc per kilogram of absolute alcohol. In all instances one hour was allowed for equilibration between the blood and tissues, or for absorption. Blood samples were taken at appropriate intervals for alcohol analysis.

The concentration of isopropyl alcohol was determined in blood and body fluids by distilling off the alcohol under reduced pressure into a solution of potassium dichromate in strong sulphuric acid. The reduced dichromate was estimated iodometrically. All reagents and procedures were identical with those as described by Newman (4) for the determination of ethyl alcohol with the exception that one cubic centimeter of N/40 thiosulphate is the equivalent of

<sup>1</sup> This report is part of the project which involves a complete pharmacologic investigation of isopropyl alcohol and is supported by the Standard Alcohol Company of New York, through courtesy of Mr. James Park.

0.7507 mgm of isopropyl alcohol. A check on the accuracy of the method as determined by analyzing definite concentrations of the alcohol in water and blood showed that 96 per cent to 104 per cent was recovered using 1 cc samples and in a range from 12 to 342 mgm. per 100 cc. All analyses of blood samples from experimental animals were made in duplicate and reasonable checks were obtained.

**RATE OF FALL IN THE BLOOD STREAM AFTER INTRAVENOUS ADMINISTRATION.** Ninety-eight observations were made on 13 dogs. The individual curves for each of 3 dogs given  $\frac{1}{4}$ ,  $\frac{1}{2}$  and  $\frac{3}{4}$  of the fatal intravenous dose and averages are presented in figure 1. The average degrees of drunkenness for each group are also shown as determined by the method of Newman and Card (5). It is seen that there is some individual variation in the blood alcohol curves, but more important is the fact that the rate of decline of isopropyl alcohol is related to the concentration in the blood. This is in contrast to ethyl alcohol which disappears at a constant rate so that the curve of blood alcohol is linear as pointed out by Widmark (6) and others. When Widmark's factor Beta or rate of fall of blood alcohol in milligrams per cubic centimeter per minute, was calculated for isopropyl alcohol it was found that this varied from 0.001 for the lowest dose to 0.003 for the highest dose. This indicates that the rate of combustion is not directly proportional to the concentration and other factors must operate to remove the alcohol from the blood. During the course of these experiments saliva, vomitus and urine were analyzed whenever specimens could be obtained. Specimens from one dog which received  $\frac{3}{4}$  of the intravenous fatal dose contained high concentrations of isopropyl alcohol. Vomitus obtained 3 hours after injection contained 311 mgm. per cent, urine samples at 1, 6 and 7 hours after injection gave values of 328, 213 and 239 mgm. per cent and saliva collected during the injection period reached 356 mgm. per cent. Thus it is evident that isopropyl alcohol is lost not only through the kidneys but is eliminated by other secretory and excretory organs as well.

The minor role of the kidneys in this excretory process was demonstrated by the following experiment. The blood alcohol was followed in 2 dogs before and after nephrectomy. The results are presented in figure 2. Dog 1 received  $\frac{1}{4}$  of the fatal intravenous dose or 1.28 cc. per kilogram and dog 2 was given  $\frac{1}{2}$  the fatal intravenous dose or 2.56 cc. per kilogram intravenously before and after removal of the kidneys. The highest blood alcohol value attained by dog 1 with the kidneys intact was 100 mgm. per cent at the end of the first hour. This fell to 34 mgm. per cent in 8 hours, or a total fall of 66 mgm. per cent. In the same animal after nephrectomy the blood alcohol reached 97 mgm. per cent by the second hour and dropped to 36 mgm. per cent in 8 hours or a fall of 61 mgm. per cent which is almost identical with the total for the normal dog.

In dog 2 the maximum blood alcohol concentration for the normal animal was 229 mgm. per cent which fell to 131 mgm. per cent in 8 hours or a total decline of 98 mgm. per cent. When the kidneys were removed the blood alcohol attained a value of 281 mgm. per cent at the end of the first hour and fell to 178 mgm. per cent in 8 hours. This was a total drop of 103 mgm. per cent which

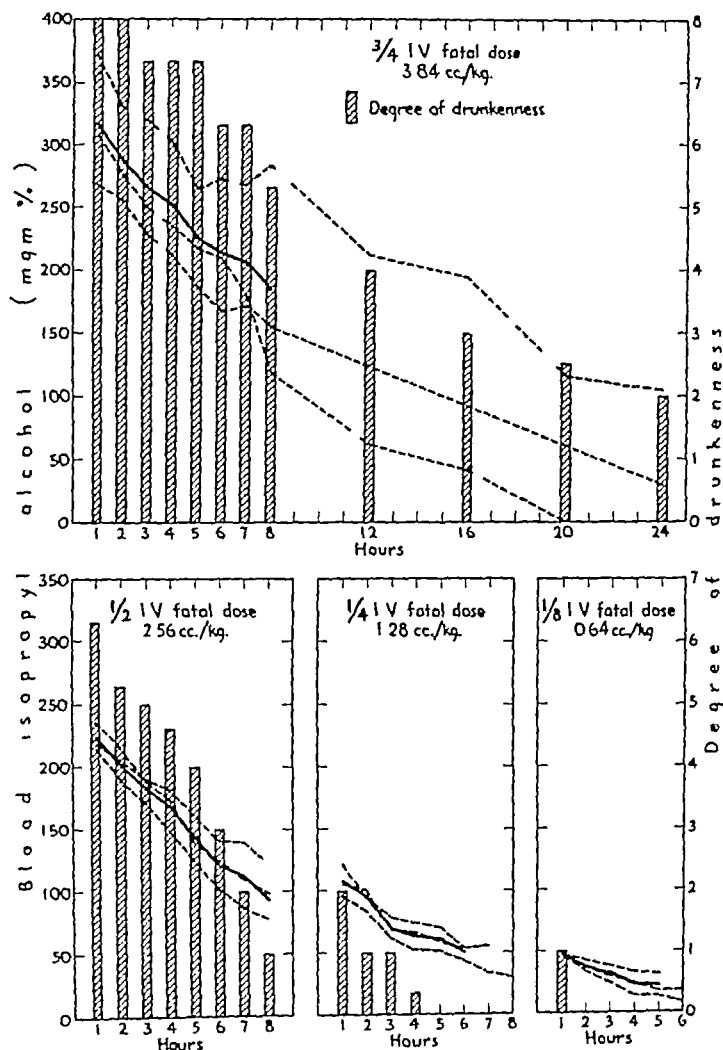


FIG 1 BLOOD ISOPROPYL ALCOHOL CONCENTRATIONS AND DEGREES OF DRUNKENNESS AS PLOTTED AGAINST TIME AFTER INTRAVENOUS INJECTIONS OF PROPORTIONATES OF THE INTRAVENOUS (IV) FATAL DOSE

The heavy line is the average of blood alcohol concentration for the 3 dogs in each group  
Degrees of drunkenness are also averages

again approximates the value for the normal dog. In both animals a higher concentration of isopropyl alcohol was maintained during the observation period after removal of the kidneys but the general slope of the curves was about the

same. This indicates that there are other channels of excretion which include the stomach and salivary glands as already pointed out.

**RATE OF FALL AFTER ORAL ADMINISTRATION** Isopropyl alcohol was administered gastrically to 9 dogs, or 3 dogs on each dosage schedule of  $\frac{1}{3}$ ,  $\frac{1}{2}$  and  $\frac{2}{3}$  the oral fatal dose (1) which represented 0.93 cc. to 3.75 cc. per kilogram of absolute

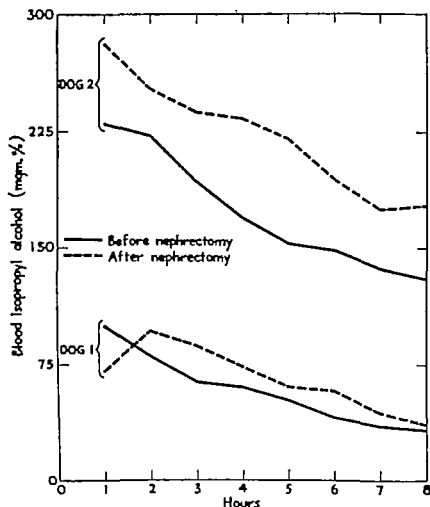


FIG. 2. RATE OF DISAPPEARANCE AND CONCENTRATION OF ISOPROPYL ALCOHOL IN THE BLOOD OF NORMAL AND NEPHRECTOMIZED DOGS FOLLOWING INTRAVENOUS INJECTIONS

Dog 1 received  $\frac{1}{3}$  of the fatal intravenous dose or 1.28 cc. per kilogram and dog 2 received  $\frac{2}{3}$  the fatal intravenous dose or 2.56 cc. per kilogram

alcohol. Figure 3 presents the individual curves for each dog. It is apparent at once that absorption, as reflected by the blood alcohol concentrations, takes place rapidly. Most of the dose is absorbed within 30 minutes and was even complete in one dog on the highest dose within that period although 1 to 2 hours were required for maximum diffusion throughout the tissues in the majority of animals. Blood alcohol concentrations varied considerably both as to groups and to individual animals in each group. These variations are greater in extent than those observed with ethyl alcohol as demonstrated by the curves presented by Newman and Card (5). A possible explanation might be that the alcohol is

absorbed but re-excreted into the stomach where it is again absorbed, creating fluctuations in the blood alcohol curves. The high alcohol content of vomitus obtained from dogs given intravenous injections of the alcohol would tend to support this assumption. The blood alcohol in the dogs which received the highest dose, or  $\frac{1}{2}$  the gastric fatal dose, approached zero at the end of 24 hours. The slope of the curve had a tendency to reach a plateau and fall more slowly as the alcoholemia reached a low level. The rather short sojourn of isopropyl alcohol in the blood does not support the contention of Morris and Lightbody (3) who suggest that the alcohol is slowly detoxified.

**INTOXICATING EFFECTS** It was previously reported that the general effects of isopropyl alcohol on the central nervous system were similar to those of ethyl

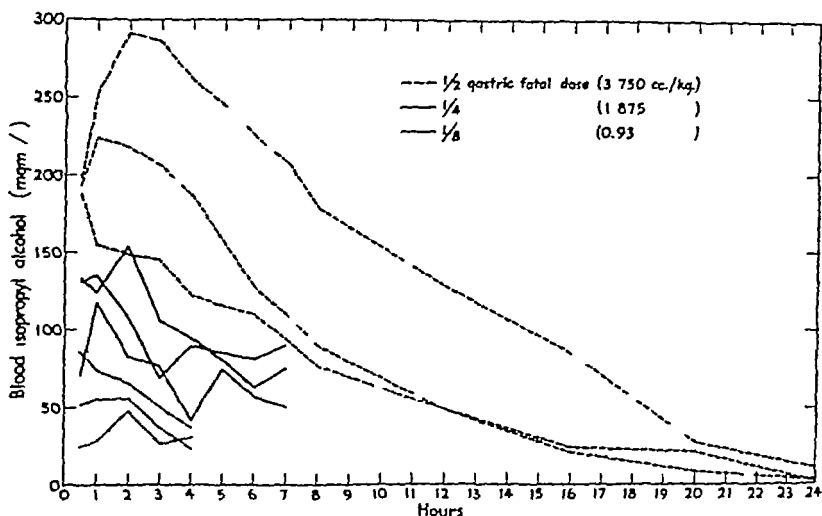


FIG 3 BLOOD ISOPROPYL ALCOHOL CONCENTRATIONS IN DOGS FOLLOWING ORAL ADMINISTRATION OF PROPORTIONATES OF THE GASTRIC FATAL DOSE

alcohol (1). To further test this similarity the degrees of drunkenness were correlated with the blood isopropyl alcohol concentrations in the dogs which received the alcohol intravenously. This is presented in figure 1. It is seen that each 50 mgm per cent of blood isopropyl alcohol is about the equivalent of 1 degree of drunkenness according to Newman's scale. Only an approximate comparison can be made with ethyl alcohol, but it may be said that blood ethyl alcohol must reach about twice that of isopropyl alcohol for comparable degrees of drunkenness. This is in substantial agreement with their relative toxicities. Metabolic by-products such as acetone have been suggested as contributing to the depressant effects of isopropyl alcohol. Qualitative tests for acetone were positive in many of the bloods and urines of the dogs used in this study but the feeble narcotic action of the ketone can scarcely be considered as a contributory factor. Quantitative studies of this phase of the problem will be reported in a separate communication.

## CONCLUSIONS

- 1 Isopropyl alcohol in blood and body fluids may be estimated iodometrically by a method similar to that employed for ethyl alcohol
- 2 The disappearance of isopropyl alcohol from the blood stream of dogs after intravenous administration of a single dose does not proceed at a constant rate and the rate of combustion is not directly proportional to the blood concentration.
- 3 The rate of disappearance of isopropyl alcohol from the blood is dependent upon the functional efficiency of all excretory organs as high concentrations were found in saliva, urine and stomach contents following intravenous administrations.
- 4 Isopropyl alcohol is rapidly absorbed from the gastrointestinal tract and distribution to the tissues can occur within thirty minutes to one or two hours.
- 5 The narcotic action of isopropyl alcohol is about twice that of ethyl alcohol

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# THE VALUE OF THE GUINEA PIG CORNEAL REFLEX FOR TESTS OF SURFACE ANAESTHESIA

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The search for tests of local anesthetic activity that could throw light on the suitability of a local anaesthetic for specific therapeutic purposes has been largely responsible for the development of the methods at present in use. They are the guinea pig wheal test (1, 2, 3), intrathecal injection into the frog (4) and the rabbit (5), and the rabbit corneal reflex test (6).

These tests suffer from two serious disadvantages. First they have not proved suitable for development even to that degree of quantitative accuracy achieved by most accepted methods of biological assay. Secondly by concentrating on overall effects two of them, at any rate, can throw little light on the exact nature of the local anaesthetic action involved. Nevertheless such tests have a real qualitative value because they reveal immediately whether or not a substance acts in the living body and also whether it produces undesirable side effects. But the anaesthetic action so revealed depends on a complex of factors, any one or more of which may affect the potency of the drug, and it is therefore, in our opinion, unfortunate that much effort has been devoted to attempting to give these qualitative tests a quantitative basis.

The wheal test in the guinea pig is in many ways the least satisfactory. The criterion of response is as indefinite as the stimulus is variable; furthermore the action of a drug like cocaine is in this test complicated by its vasoconstrictor effect which can be more accurately studied separately. The method of intrathecal injection into the frog has hitherto been the best available for assessing quantitatively the effect of local anaesthetics on myelinated nerves. It has now been replaced on the one hand by Bennett's (7) test and on the other by the rabbit test of Bieter (5) *et al.* for spinal anaesthesia and is thus no longer of much interest.

Bennett introduced his test to measure reversible interference with nerve impulses by the reduction of the  $\alpha$ -wave action potential in an isolated myelinated nerve. The method is not only suitable for assessing the rate at which the anaesthetic penetrates through the nerve sheath—an important factor influencing both intensity and duration of local anaesthesia at different sites in the body,—but also constitutes a successful attempt to measure the fundamental pharmacological action of local anaesthetics. The conditions of the test can be rigidly controlled unlike those carried out on the living animal which need statistical evaluation. The property studied by Bennett undoubtedly plays its part when a local anaesthetic acts *in vivo* but there is, in our opinion, still need for tests of infiltration and surface anaesthetic action on the live animal capable of some degree of quantitative accuracy. Bieter's test, well suited for its specific

purpose, is only legitimately to be used for the investigation of spinal anaesthetics

Several workers (8, 9, 10) have compared the local anaesthetic activity of closely related chemical compounds by the methods discussed above, but no attempt has been made in them to evaluate the activity with greater accuracy than that achieved by arbitrary methods of scoring. For such comparisons, now that the study of local anaesthesia has become a branch of chemo therapy, quantitative methods of *in vivo* test are urgently required. It is generally recognised that the action of a local anaesthetic, like that of many other drugs, frequently involves penetration of a biological interface, and conditions for studying this would appear particularly favourable in the cornea. This accounts for past attempts to use suppression of the corneal reflex in the rabbit as a measure of surface anaesthesia. Unfortunately, although this is the only method developed for the purpose, it has not been successfully standardised and has not withstood statistical examination.

The comments of other workers support what we have said about the accuracy of the wheal and corneal tests.

H K Sinha (1 and 2) states that "a comparison of the activity of two anaesthetics can be obtained from three or four experiments in the case of the rabbit's cornea or the human wheal" but that "in general a high degree of accuracy cannot be obtained with these tests and they are chiefly serviceable in measuring gross differences in activity."

T H Rider (3) comments in much the same way on the corneal reflex of the rabbit, when he concludes that "satisfactory standardisation of compounds can be made only on the group of rabbits which react normally" since at least one half of the rabbits tested have given a cocaine anaesthesia 10 to 15 minutes shorter than the average value of 27 minutes."

The cornea, however, provides the ideal site for the measurement of surface anaesthetic action, for it is free of special sensory cells, the nerve endings are free, unsheathed and imbedded in the layers of the cornea and the drug consequently has a single uniform membrane to penetrate, after which it is in direct contact with sensory nerve fibers. The cornea is, moreover, free of fluid channels and blood vessels.

MacIntosh and Work (9) used the corneal reflex of the guinea-pig to investigate the local anaesthetic activity of ammoethyl derivatives, but gave no indication of the accuracy of their method.

Preliminary investigations satisfied us that, as found by MacIntosh and Work (private communication), guinea-pigs show a more regular reflex response, both normally and when anaesthetised, than do rabbits. We then carried out a number of experiments based on Latin square designs, with a view to detailed statistical analysis of the responses shown by each species to each of three different local anaesthetics.

Local anaesthetics may be compared for two properties, effective concentration and duration of effect. These two properties may be, and in practice often are, related. Nevertheless it is valuable to distinguish between them when

assessing the properties of local anaesthetics. In this paper we are primarily concerned with the assessment of surface anaesthetic potency and not of the duration of action

The three local anaesthetics used in this investigation were the hydrochlorides of cocaine which we have regarded as a 'standard' amethocaine (*p*-butyl aminobenzoyldimethylaminoethanol) and procaine (B P). We have studied the median effective doses (ED 50) of the three anaesthetics on both species as well as the slopes of the dosage-response curves. We have related these values to their standard errors for different periods after administering the drug for two different dose ratios and for left and right eyes considered separately and we have also taken into account any effect of one dose on the animal's response to a later dose. It may be said here that we have been unable to verify T. H. Rider's statement (3) that such an effect is produced by cocaine hydrochloride. This will be made clear when we report elsewhere details of the statistical analysis. Further no significant difference could be found between the left and right eyes of guinea pigs.

Examination of the six tests showed that the probit percentage responses as defined below, bore to log doses a linear relation over a remarkably wide range and that there was no significant departure from linearity. Furthermore the slopes themselves were highly significant, in each instance the ratio of  $b$  to  $\sigma_b$  was over 9.

Consideration of the time relationship led to the standardisation of technique in the manner described below where we give precise details of the method used in carrying out an estimate of the potency ratio of two different local anaesthetics. Conditions for testing the surface anaesthetic potency of a local anaesthetic are a constant minimal stimulation combined with a suitable fixed interval of time. The procedure used for these investigations consists of instilling a quantity of the anaesthetic dissolved in saline solution under the eyelid and recording the presence or absence of the reflex blink after suitable stimulation is applied to the surface of the cornea.

The cornea was stimulated once a minute for a fixed period of time after instillation the response was recorded as the number of times the animal failed to blink as a result of the stimulus. The period used for the comparison of the rabbit and the guinea pig was fixed at five minutes permitting five separate stimulations to be made. The suitability of this period of time was established by plotting the total number of responses occurring at minute intervals after instillation of the local anaesthetic in concentrations that induced the whole range of responses. It is seen that the maximum effect is induced two minutes after the application of cocaine hydrochloride to the guinea pig cornea and five minutes afterwards in the rabbit. No appreciable diminution of the effect occurs in the guinea pigs until after five minutes however and this was therefore chosen as the most suitable interval of time during which to compare effects on the two species.

It is clear that an increase in the number of observations which ordinarily diminishes the error of the mean observation of a group may in tests on local anaesthetics be accompanied by a falling off in the effect of the anaesthetic if

this effect has previously reached a maximum. The best time at which to end readings depends, therefore, on a compromise between two opposing factors.

It is possible that the accuracy of the test could be increased by increasing the number of observations in a given time, though it is doubtful whether much more than a 50 per cent increase in this number could in practice be made, and this would not have a very marked effect on the standard error of the mean. Furthermore, as we hope to demonstrate, the error of this test is so low, owing to the

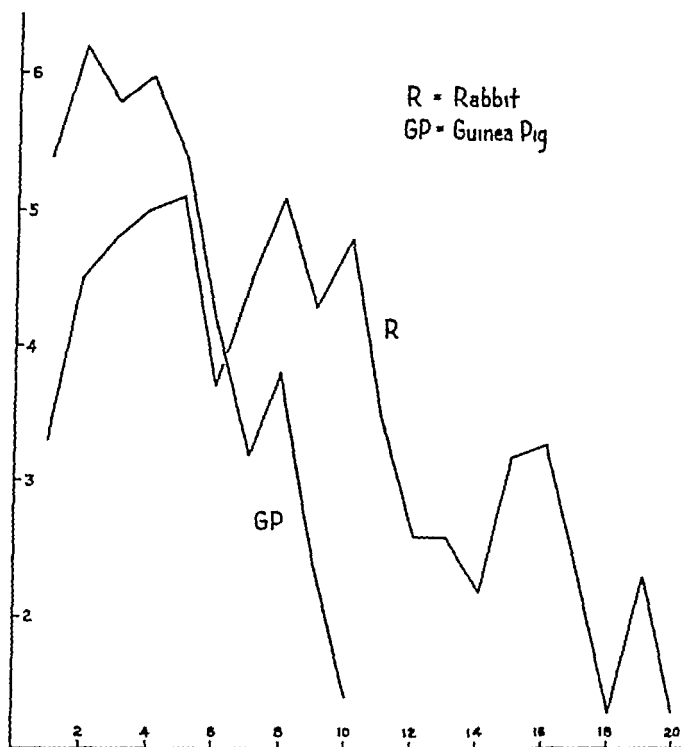


FIG 1 EFFECT OF TIME ON CORNEAL RESPONSE TO COCAINE HYDROCHLORIDE  
Vertical axis: Average number per minute of "responses" (see text) for six different doses to six animals. Horizontal axis: Time (in minutes) after instillation.

unexpectedly regular behaviour of guinea pigs, that we believe it to have nearly reached the limits imposed by biological variability.

**NATURE OF STIMULUS** A constant stimulus was applied by a horsehair mounted in a glass rod. This was pressed against the center of the cornea in such a way that the hair was bent approximately to the same extent at each application.

**VOLUME OF DOSES** Lesser (6) demonstrated that the optimal anaesthesia measured in terms of the product of the duration time and threshold rise in volts,

using electrical stimulation was obtained with a minimal quantity of 0.8 ml of the solution containing the anaesthetic but he does not say for how long this quantity was allowed to remain in contact with the eye

Sinha (2) also maintains that the quantity of the dose is important. He states that small amounts applied to different parts of the cornea give different results and that therefore total flooding of the eye is necessary. He leaves the solution in contact for five minutes and states that the infero-posterior quadrant is more trustworthy than other parts of the cornea.

The methods used by these authors involve the expenditure of a considerable amount of time and are sometimes complicated. We therefore decided when making the comparison of the two species for suitability as test animals to use a technique that would not involve excessive delay in applying the drug. The guinea pig unlike the rabbit has only a small space between the lids and the cornea, and it was therefore found necessary to use the amount that could be applied directly to the surface while holding the lower lid free to allow the fluid to run down between the lid and the cornea. This was for the guinea pig approximately 0.05 ml.

**DESCRIPTION OF PROCEDURE** Six adult guinea pigs are arranged in a row on the bench facing the operator. Standard bench clamps are supported on a horizontal bar above the guinea pigs. Each guinea pig is held lightly by these clamps which exert pressure on the flanks and slightly compress the abdomen posterior to the costal margins. On the first occasion the animals may be somewhat restless but they will soon become accustomed to the treatment and will thereafter remain quiet.

The solutions containing the different concentrations of standard and test substances are arranged in vials next to the guinea pigs and are taken up into a dropping tube from which they can be applied rapidly to the surface of the cornea. This is done by allowing a drop to run on to the corneal surface so that the space between the eyelids contains a clearly visible film of the solution. This can be repeated several times without causing the animal to blink as a result of the application and ensures that the fluid remains in contact with the cornea for at least two seconds. If it is expelled instantaneously by a reflex blink then a second or if necessary a third application is made. The first stimulant is applied to each eye after the lapse of approximately 45 seconds from instillation. The stimulus is then repeated each minute on each eye.

The number (and percentage) of responses—that is *failure* to exhibit the reflex blink—is recorded for each animal and each dose, the two eyes being taken as identical so that ten observations are obtained during a five minute period for each animal and each dose. The mean percentage response for each dosage group is recorded and the standard errors of the probits of these mean responses are calculated in the usual manner—by variance analysis if a factorial distribution of doses and animals has been used or by use of the mean variance of a single observation assuming variance to be independent of response level.

For calculating the values of the median effective dose or of potency ratio when two or more substances are being compared in a simultaneous test we use the ordinary probit percentage as the measure of response and the logarithmic dose as the controlled variable. Calculations follow by the usual modification of the method of least squares such as those described by Irwin (11). The error of the test can be calculated by the method given in Irwin's paper when the ratio of  $b$  to  $s_b$  is over 5 otherwise the more precise formula for fiducial limits is better employed. In this connection it is worthy of note and somewhat remarkable that we have never found any significant difference between the guinea pigs used in this test. Since the order of dosage also makes no significant contribution to the

error of test, a very simple experimental design can be used, provided the stock of animals has been found to show the same uniformity in response as occurred during our experiments. No strict balancing of doses against animals or order of administration is necessary.

The proof of any biological test method lies in the assay. Having made a provisional comparison between the activities of three anaesthetics on both guinea-pigs and rabbits, and having selected the former animal as more suitable for assay purpose, we therefore carried out an independent test on six previously untreated guinea-pigs, comparing the three anaesthetics each at two dose levels, intended to lie on each side of the ED 50 as estimated earlier, when investigating the dosage response relationships. The results of these tests are given in table 1.

TABLE 1

*The Median Effective Concentrations, E (in milligrams per millilitre of aqueous solution) of the Hydrochlorides of Amethocaine, Cocaine and Procaine as Surface Anaesthetics*

	AMETHOCAINE		COCAINE		PROCAINE	
	I	II	I	II	I	II
E	0.68	0.56	2.15	2.63	35.5	26.0
True fiducial limits of E, for, P = 0.95	0.57-0.83	0.30-0.67	1.85-2.48	1.73-3.51	29.5-45.2	12.6-29.0
Slope of dosage response curve ± its standard error	2.03 ±0.19	" under cocaine	2.74 ±0.23	2.14 ±0.445	1.62 ±0.17	" under cocaine

The series I refers to the separate determinations of dosage-response relationships: the experiments with cocaine were carried out five weeks before those with procaine and six weeks before those with amethocaine. The series II refers to the "six-point" assay carried out contemporaneously (some eight months later) on all three compounds. In this series the value of *b*, and its standard error, is the same for all three substances because it was got from all six groups of animals, statistical analysis having shown that the three contributory values of *b* did not differ significantly ( $\chi^2 = 3.3$ ,  $n = 2$ ,  $p = 0.19$ ).

For purposes of comparison we have also included the figures obtained during the earlier and more detailed investigation. It will be seen that the agreement is on the whole good. We are satisfied that the "six-point assay," carried out by direct comparison of the three substances, gives the more accurate estimate of their relative activities, because the doses were given and the responses were evoked more or less contemporaneously. On the other hand, owing to the exploratory nature of the earlier investigation into the dosage response relationship, the experiment with procaine was done some five weeks later than that with cocaine, which was followed a week later by the experiment with amethocaine.

In view of what has been said at the beginning of this paper, it is not surprising that accurate statements as to the relative "activity" of different local anaesthetics are not easily to be found in the literature. It is believed that the work here reported represents the first attempt to evaluate in reasonably quantitative terms a particular type of local anaesthetic activity shown by three different compounds. It will be seen that procaine, as is well known, is much less active—

that is it is effective in a given time only at a much higher cocaine the potency ratio being approximately 1 to 10. cocaine as one had reason to believe from the literature, i.e. in the defined sense, than cocaine. In the six point test to be 4.7 to 1. In view of this fact we regard vague generalization that 'amethocaine is ten times as active as cocaine' as no foundation, but we are satisfied that in its effect on the guinea pig at any rate amethocaine is between four and five times as effective which is in turn between fifteen and twenty times as effective as cocaine.

In comparing the usefulness of drugs for a particular purpose it is desirable to compare their therapeutic indices that is to effective doses. This index may be given a more definite meaning by defining it as the ratio of the median toxic dose (LD 50) to the median effective dose (ED 50), effectiveness being considered in terms of a particular physiological property. Unfortunately as with these local anaesthetics it is difficult or impossible to determine values of both LD 50 and ED 50 for species of animals. The toxicities of amethocaine have already been evaluated for mice in these laboratories but the corresponding values for guinea pigs. Similarly, values are available only for guinea pigs and rabbits and those for the latter are of doubtful validity. It is of doubtful validity to argue over closely established for one species to the probable index for another. It is needed in transferring the value of a therapeutic index from one species to yet a third. Nevertheless if we wish to get some idea of the relative safeties of these three local anaesthetics to men we have mice and guinea pigs at our disposal.

If then with these provisos in mind we define the *relative safety* of compounds as the ratios of the LD 50 for mice to the ED 50 for guinea pigs remembering also that the former is expressed as unit weight and the latter as unit weights per unit volume, then we make on this basis a comparison of the relative safeties. Bacharach & Middleton (12) found the relative toxicity of cocaine to procaine to be 4.5 to 3 to 1 by the intravenous route. The relative values of LD 50 as A C P = 200 300 900. If we have found the relative values of ED 50 to be A C P = 10.3 3.3 1. Thus amethocaine would appear to be three times as safe as cocaine and some ten times as safe as procaine.

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# THE EVALUATION OF THE ASSAY OF VITAMIN P BY MEANS OF THE EFFECT OF LOW PRESSURE ON MICE<sup>1</sup>

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About two years ago Majovski Lesser, Lawson, Carne and Thienes (1) devised a method for the assay of vitamin P in various preparations.<sup>2</sup> The test consisted of exposing mice for one minute to a low atmospheric pressure and then examining them for signs of pulmonary hemorrhage. While nearly all of the animals died, it was claimed that in those previously fed preparations containing the supposed vitamin the hemorrhage was not so extensive as in the controls.

We have followed the original experimental procedure and have observed that a certain type of injury developed in the lungs of mice that had recently taken food whereas it did not appear in the lungs of animals that had been deprived of food. These results will be presented in detail in a later publication. In the present paper we wish to discuss the nature of the injury to the lungs observed in mice suddenly exposed to low pressure and the supposed effect of previous feeding with various preparations upon the incidence of such injuries.

Upon gross examination the lungs of some of the mice that succumbed to the sudden reduction in pressure had a hemorrhagic appearance while those of others were pale and, except for occasional small areas, non-hemorrhagic in appearance. Microscopic examination by Dr S Wilens to whom we are greatly indebted for permission to report his findings disclosed the following:

The lungs which upon gross examination had a hemorrhagic appearance showed the following changes. The air spaces were almost completely collapsed. The alveolar septa were folded and approximated against each other. They were relatively thickened and their capillaries increased in diameter and filled with blood. In the interstices of these collapsed alveoli i.e. the remnants of the alveolar spaces a few extravasated erythrocytes could be seen. In some instances the amount of free blood was greater and the lungs were studded with petechial hemorrhages but there was never massive hemorrhage and the bronchi never contained blood. The larger blood vessels were markedly engorged.

The lungs which upon gross examination were pale and non hemorrhagic in appearance differed from those in the preceding group in that the alveoli were still expanded. Small areas of atelectasis were sometimes encountered and these resembled the tissue described above. For the most part however the alveolar sacs were distended with air. The alveolar walls were thin. The alveolar capillaries were compressed and relatively bloodless. Free blood in the air passages

<sup>1</sup> This research was supported by The California Fruit Growers Exchange Research Fund of the College of Medicine New York University.

<sup>2</sup> We wish to acknowledge our indebtedness of Dr C H. Thienes for furnishing us with the details of this work prior to its publication.

was only rarely seen. A few of these animals showed areas of pulmonary edema. The larger blood vessels were similar to those in the first group. Neither group showed traumatic changes such as rupture of alveolar septa."

Regardless of whether the lesions observed were true hemorrhages, their incidence might have been affected by the previous administration of preparations of the supposed vitamin. We did not find this to be the case. Possible reasons for the difference between our results and those of Thienes and his associates will be discussed later.

**EXPERIMENTAL** White mice varying in weight from 15 to 18 grams were used. Males and females were kept separately, but sex, apparently, did not influence the results. We used four different strains of mice, which were purchased from four different dealers. They were fed on the same commercial prepared food (Fox Food Blox).<sup>3</sup> The source of the animals seemed to have no effect on the results of the tests, nor did such variations in room temperature as we encountered (20-27°).

Three series of experiments were conducted. In Series 1, the mice were taken directly from stock cages containing food. In Series 2, the animals had fasted over night. In Series 3, we used previously fasted mice which had been allowed to feed for one hour on the commercial food. To the mice from all three series, the experimental material was administered by stomach tube, either in solution, or in suspension. The animals were then placed in cages where they had access to water but not to food. The tests were then made after from one to two hours had elapsed.<sup>4</sup> We used, as controls, mice which had received 0.9 per cent sodium chloride and mice which had received no fluid by stomach tube. The results obtained with the two kinds of controls were essentially the same, and were combined.

The apparatus consisted essentially of four parts: a vacuum pump, two empty commercial gas tanks of about 30 liters combined capacity, a vacuum manometer and a small desiccator. These were connected by pressure tubing. To operate, the pressure in the tanks was adjusted to 30 mm. of mercury, with the desiccator shut off from the rest of the system by means of a three-way stopcock. Usually six mice were then placed in the desiccator, and the stopcock was opened to the reservoir tanks. About 15 seconds were required for the pressure in the entire system to come to 70 mm. At the end of one minute, air was readmitted to the desiccator. Atmospheric pressure was reached in about five seconds. The mice were then allowed to remain in the desiccator for one minute longer.

About one per cent of the animals survived the tests and were arbitrarily assumed to have sustained no injury to the lungs. The dead mice were examined immediately. We used two methods for evaluating the extent of the lesions, both based upon a gross examination of the pleura of the lungs. In Method 1, which is that of Thienes and his associates, the percentage of altered tissue was estimated, and the average for each group reported. Since the lungs fell into

<sup>3</sup> Allied Mills, Inc., Chicago, Ill.

<sup>4</sup> In the work of Thienes et al. it is reported that the most marked effects were obtained one and two hours after administration.

two fairly distinct groups one with small amounts of visible lesions and the other with large amounts, it seemed to us to be more valid to designate the results either positive or negative. In Method 2 we have taken as positive those lungs in which at least 50 per cent of the pleural surface showed changes. In order to show the large number of lungs in which 100 per cent was injured, we list this separately in the table.

TABLE 1

MATERIAL	DOSE	TIME AFTER ADMINIS- TRATION	NO OF ANIMALS	METHOD 1	METHOD 2			
				% Injury	Positive	100 % Injured		
Not fasting (series 1)								
	mg.	ml	hrs.			N	%	
Crude hesperidin	40	0.5	1-2	13	98	13	100	11
Chalcone	10	0.2	1-2	12	94	12	100	9
Methyl chalcone	40	0.4	1-2	9	80	9	100	7
Controls			1-2	32	65	23	72	16
Fasting (series 2)								
Chalcone	20	0.4	1-2	18	40	9	50	3
Methyl chalcone	20	0.4	1-2	13	26	3	23	2
Controls			1-2	30	13	1	3	0
Fasting plus 1 hour on food (series 3)								
Crude hesperidin	40-48	0.5-0.6	1	24	85	21	87	14
			2	12	83	10	83	8
Chalcone	48	0.6	1½	12	93	12	100	11
Methyl chalcone	30-48	0.3-0.6	1	15	83	13	87	9
			2	18	93	17	94	12
Eriodictin A52	20-40	0.5	1	40	92	26	90	30
Sodium hesperidinate	30-48	0.3-0.6	1	6	98	6	100	5
			2	18	98	18	100	15
Controls			1	31	96	30	97	25
			2	18	81	16	89	11

We tested the following preparations

- 1) Crude hesperidin, obtained from Abbott Laboratories
- 2) Hesperidin methyl chalcone obtained from The California Fruit Growers Exchange
- 3) Eriodictin A52 also obtained from The California Fruit Growers Exchange
- 4) Sodium hesperidinate\* prepared by treating hesperidin recrystallized from pyridine with sodium hydroxide. This material is probably a mixture of the sodium salts of hesperidin and of its chalcone
- 5) Hesperidin chalcone\* prepared by treating the recrystallized hesperidin with 1N NaOH at 100°

**DISCUSSION** The results are summarized in Table 1. In Column 6 is shown the average percentage of lung tissue which was injured (Method 1). In Column

\* We are indebted to Dr. W. E. Baler of the Research Department of the California Fruit Growers Exchange for furnishing us with the methods of preparation.

7, we give the number of animals showing the lesions which we have designated as positive (at least 50 per cent injured), and in Column 8, the percentage of animals in which the lungs were so changed. The last column shows the number of lungs in which 100 per cent of the pleural surface showed changes. We believe that Method 2 leads to a more reliable evaluation although, in the present work, the results obtained by the two methods are in remarkable agreement.

The results in Table 1 indicate that none of the materials which we have investigated significantly reduced the degree of injury to the lungs produced in the mice exposed to low pressure.

We have also made some tests with mice which were given the vitamin P preparations both intraperitoneally and intravenously. Other tests were made with animals which had been previously injected intraperitoneally with daily doses of "eriodictin A52" for one week. In neither experiment was there any indication of increased resistance to the low pressure under the conditions of the test.

Dr Thienes now writes that not all preparations of crude hesperidin were protective, in fact, that only one of several specimens had any such action. He also writes that, whereas one specimen of eriodictin, labelled A52, was protective in high degree, another sample, also labelled A52, afforded little protection. It may be that the differences in our results are due to the varying content in the various preparations of some unknown constituent to be known as Vitamin P, or to the very slight differences in the time required to obtain a pressure of 70 mm, or to a combination of both of these. We believe that such slight differences as actually were obtained were due to quite other causes. As is made evident in the following paper, fasting exerts a "protective action," and since in the experiments of Thienes et al, the intake of food was not controlled, it may very well be that the slight differences observed were due to the failure of the animals receiving the various preparations of hesperidin et cetera to take as much food as did the controls. Moreover, we regard the differences between the results obtained by Thienes et al in the various groups of animals to be altogether too slight to be significant.

Accordingly, we conclude that the method proposed by Thienes et al is not suitable for the assay of vitamin P.

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# THE EFFECT OF THE ADMINISTRATION OF SODIUM BROMIDE TO PREGNANT RATS ON THE LEARNING ABILITY OF THE OFFSPRING

## II MASE-TEST<sup>1,2</sup>

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The purpose of this investigation is to study in white rats the effects of the prenatal administration of sodium bromide by means of tests designed to detect functional damage in the central nervous system. Although the changes in the adult nervous system produced by bromides are generally considered reversible quantitative data on this problem are meager (1) and except for a short report by the authors (2), the literature contains no data on the effects of bromides on the development of the embryonic and infant nervous systems. The work presented at this time deals with an analysis of the effects of the prenatal administration of sodium bromide on the mase-learning ability of rats. The rats were bromide-free at the time of the tests.

**EXPERIMENTAL ANIMALS** *Groups* Four groups of albino rats were used in these experiments three experimental groups representing three levels of the factor being studied and a control group. All animals were bred from the Wistar Experimental Strain of the stock maintained by the Department of Pharmacology of the Woman's Medical College of Pennsylvania. The 120 group consisted of 34 offspring (18 females 16 males) of females which had been administered by stomach tube daily 120 mgm. of sodium bromide per kgm. of body weight from the third through the twentieth day of gestation. The '80 group consisted of 80 offspring (12 females 18 males) of females which had been dosed similarly during the same period of pregnancy with 80 mgm. of sodium bromide per kgm. per day. The 40 group consisted of 33 offspring (13 females 20 males) of females dosed for the same period with 40 mgm. per kgm. per day of sodium bromide. In the experimental groups the rats were born approximately two days after their mothers had received the final dose of the drug. The control group of 80 animals (13 females 17 males) were offspring of females which had received no sodium bromide during pregnancy. These mothers were normal in all known respects.

*Routine* 1 After birth the young rats received no bromide except that obtained from the milk of their mothers. All of the animals were weaned at 20 days of age unless the mother started to destroy the litter earlier. In the bromide groups '80 and 120 several litters were weaned at 14 and 15 days. For a few days the growth of these animals was sub-normal but the handicap was temporary and of a minor order. Weight curves on these rats reveal no evidence of any physical handicap.

<sup>1</sup> This investigation has been made with the assistance of (1) Grant No. 567 (1940) from the American Philosophical Society (2) Grant No. 462 (1941) from the Committee on Therapeutic Research Council on Pharmacy and Chemistry American Medical Association (3) Grant No. 1 from the Department of Neurology of the Woman's Medical College of Pennsylvania.

<sup>2</sup> The authors wish to acknowledge their indebtedness to Silvia Fishbein Yaffe and Dorothy Moesta for technical assistance.

2 From age 20 through 34 days the drinking water was replaced by a 0.2 per cent solution of sodium chloride and from age 35 through 41 days by a 0.5 per cent solution

3 At 43 days of age the rats were delivered to the laboratory of psychology in order that they might become adjusted during the following two weeks to their new environment and attendants

4 From age 57-60 days the rats were prepared for learning in the maze

5 From age 61-85 days each animal was given two trials per day in a five cul-de-sac U-maze

**Diet** The mothers during the period of pregnancy, and the test-rats through 42 days of age, were given free access to Purina dog chow checkers, powdered whole milk and brewers' yeast. Supplements of lettuce and cod liver oil were given to the mothers weekly and to the test-rats at 1, 2 or 3 day intervals, depending upon their age. After 42 days of age the diet consisted of 100 per cent whole wheat bread and milk daily, with cracked corn added on alternate days

**Bromide Determinations** The Brodie-Friedman method (3) was used for tissues and the Friedman modification (4) was employed for blood. Representative newborn rats from the control and the bromide litters were dried and analyzed for bromine. At 62 days of age blood bromide determinations were made on representative animals. These rats were not used for the psychological tests

1 *Concentration of Bromide in Mothers* One noticed that the pregnant bromide-rats offered less resistance to the passage of the stomach tube than did the normal rats but when they were observed in their cages one could not distinguish with certainty any evidence of depression. Objective determinations of activity were not made. The best measure of the bromine content of the mothers was obtained from the analyses of the new-born rats. These analyses, table 1 (dry tissue) show that the ratios between the bromine content of the control and of the experimental groups are 1 to 40 for group "120", 1 to 21 for group "80" and 1 to 11 for group "40". The difference between any pair of groups in either the wet or the dry series is statistically significant (table 2). Multiplying these relative concentrations by the mean value for serum bromine in our control rats, 3.8 mgm per 100 cc, table 3, one calculates that at parturition the values for bromine in the blood serum of the mothers were 152 mgm per cent in group "120", 80 mgm per cent in group "80", and 42 mgm per cent in group "40". These calculations would appear to be valid since bromides are confined principally to the extracellular water (5, 6) in which they are evenly distributed except for a small differential between plasma and cerebrospinal fluid (7, 8). A few determinations on the serum of mothers who lost their litters agree reasonably well with our calculated values. Similar values in the human are associated with sedation or mild depression.

2 *Concentration of Bromide in Newborn and Infant Rats* The newborn rats used for the bromide analyses were taken from the mothers approximately 4 hours after birth. These analyses showed that the bromide concentrations in the three treated groups bore approximately the same quantitative relationship to each other as the dosages administered to the mothers. Thus the bromine content of the dried tissue of group "40" is 0.53 of that of group "80" and 0.28 of that of group "120" (table 1).

The mortality among the unweaned bromide rats was high and paralleled the

does given the mothers. In table 5 we have recorded as "infant mortality" the still births and deaths before 20 days of age in the control and experimental groups. These data expressed in per cent were 2.3 for the control group, 58 for the bromide group 120, 42 for group "80" and 27 for group "40". Table 6 shows that the differences among these percentages are significant. The loss

TABLE 1  
*The bromine content of new-born rats*

GROUP	N	MG. OF BROMINE PER 100 GRAMS			
		Wet tissue		Dry tissue	
		Mean	S.E. <sub>mean</sub>	Mean	S.E. <sub>mean</sub>
Control	3	1.22	0.08	11.83	0.95
40	6	18.90	1.94	182.22	14.03
'80	8	32.19	3.11	250.40	21.47
'120	5	61.58	2.09	469.00	27.15

These figures computed on data from one less animal

TABLE 2  
*Comparison of groups shown in table 1*

GROUPS COMPARED	DIFFERENCE BETWEEN MEANS	S.D. <sub>mean</sub>	t	P <sup>a</sup>
Wet tissue				
120 — '80	29.39	4.73	6.21	<0.01
'120' — 40	42.68	2.91	14.52	<0.01
'120' — Control	60.36	2.48	24.34	<0.01
'80 — 40	13.29	3.99	3.33	<0.01
80 — Control	30.97	5.26	5.89	<0.01
40 — Control	17.68	2.84	6.23	<0.01
Dry tissue				
120 — '80	218.60	34.61	6.32	<0.01
120 — 40	336.78	29.01	11.61	<0.01
'120' — Control	457.17	36.21	12.63	<0.01
'80 — 40	118.18	27.77	4.26	<0.01
'80 — Control	238.57	36.26	6.58	<0.01
40' — Control	120.39	20.55	5.86	<0.01

<sup>a</sup>Probability that the difference obtained could occur by chance

in groups 80 and 120 would have been considerably higher except for the vigilance of the technician in weaning a litter at the first indication that the mother had started to destroy it. In this table we have recorded also the 'infant mortality' in our main rat colony for the period of the bromide studies. These animals received only routine care and a less satisfactory diet but the 'infant mortality' expressed in per cent was approximately two-fifths of that

of group "120," one-half of that of group "80" and questionably less than that of group "40" (tables 5 and 6) There were no deaths in any group between weaning and the end of the maze-tests

3 *Absence of Bromide in Animals of Experimental Age* Representative rats from the control and from each of the bromide groups were bled from the heart

TABLE 3  
*Bromine content of blood in 62 day old rats*

GROUP	N	SERUM BROMINE IN MCM. PER 100 CC.	
		Mean	S.E.-mean
Control	9	3.77	0.20
"40"	5	2.96	0.47
"80"	4	2.70	0.40
"120"	5	2.50	0.38

TABLE 4  
*Comparison of groups in table 3*

GROUPS COMPARED	DIFFERENCE BETWEEN MEANS	S.D.-diff	t	P
Control—"40"	0.81	0.43	0.18	<0.10 >0.05
Control—"80"	1.07	0.39	2.74	<0.02 >0.01
Control—"120"	1.27	0.39	3.26	<0.01
"40"—"80"	0.26	0.64	0.41	0.70
"40"—"120"	0.46	0.61	0.75	<0.50 >0.40
"80"—"120"	0.20	0.56	0.36	<0.80 >0.70

TABLE 5  
*Effect of sodium bromide on infant mortality*

GROUP	NO OF LITTERS	TOTAL NUMBER BORN	TOTAL NO LOST BEFORE WEANING	PER CENT LOST	S.D.-percentage
Control	6	43	1	2.3	2.29
Control*	24	168	37	22.0	3.20
Bromide "40"	7	63	17	27.0	5.59
Bromide "80"	22	155	65	41.9	3.96
Bromide "120"	14	119	69	58.0	4.52

\* Rats born in colony during period of experimentation These rats were given only routine care

and killed at approximately 62 days of age The serum bromine was the same in each of the bromide groups (tables 3 and 4) and all of these showed values approximately 1 mgm per 100 cc less than that of the control group A difference of this order is unimportant Possibly, it reflects the effect of a slightly greater consumption of the sodium chloride containing water which was administered *ad libitum* from age 20 through 41 days These data show that the

results obtained in the psychological tests which were started at 61 days of age were not due to abnormal quantities of bromides in the test-rats

TABLE 6  
Comparison of groups shown in table 5

GROUPS COMPARED	DIFFERENCE	S.D. DIFFERENCE	$\frac{\text{DIFFERENCE}}{\text{S.D. DIFFERENCE}}$	CHANCES† IN 100
120 — 80'	16.1	6.01	2.68	99.6
120 — 40	31.0	7.19	4.31	100
120' — Control	36.0	5.54	6.50	100
120 — Control	55.7	5.07	10.99	100
'80 — "40'	14.9	6.86	2.17	98.2
'80 — Control	19.9	5.09	3.91	100
'80' — Control	39.6	4.58	8.65	100
40' — Control*	5.0	6.44	0.78	78
40 — Control	24.7	6.04	4.09	100

Rats born in colony during period of experimentation. These rats were given only routine care

† Chances of a true difference

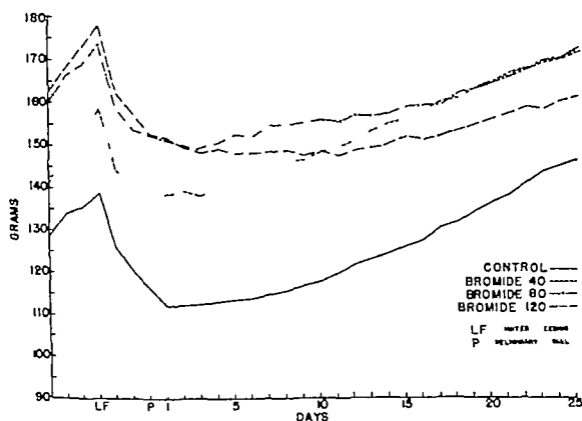


FIG 1 MEAN WEIGHT PER DAY—MALES

*Growth* The weight curves, figs 1 and 2 show the mean weight per day of males and females separately for each of the four groups for the 7 days preceding and for the 25 days of the regular maze-test. The first four days show

growth in all groups, but the "40" is heavier than the control group, and the "80" and "120" groups are alike but heavier than the "40". Beginning with the day marked "LF," the animals were allowed just fifteen minutes of feeding each day. This resulted in a loss of weight in all groups for several days, followed by a slow gain. During the loss of weight the relationship among the groups remained the same. The relationship between the control and "40" groups remained essentially constant throughout the entire period. The "80" and "120" groups gained more slowly, so that all bromide groups show nearly the same weight at the end of the maze running period, but all were heavier than the control. The explanation of this accelerated growth in the bromide

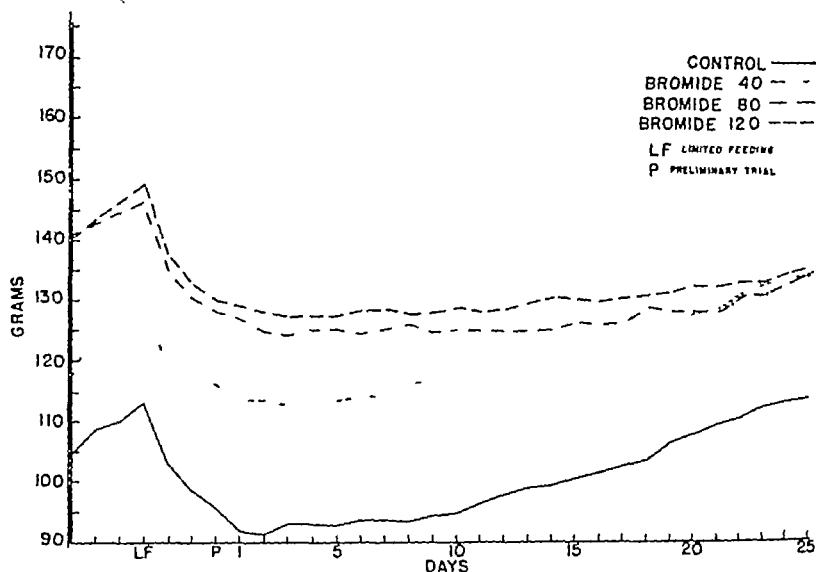


FIG 2 MEAN WEIGHT PER DAY—FEMALES

groups is not obvious, unless it is due to the natural selection which accompanied the high "infant mortality" in these groups.

**MAZE LEARNING Apparatus** The apparatus was a five cul-de-sac U-maze consisting of interchangeable units. The true pathway pattern consisted of the following turns, rrlrr. Reliability coefficients as found by previous experiments ranged from 0.84 to 0.98 (unpublished data). The units had wooden sides (painted gray) and wire mesh tops and the whole rested on battleship linoleum. The observer watched the animals through a one way vision screen. Illumination was by electricity and was constant. Hunger was the motivating factor, and food was the incentive.

**Procedure** On the fifty-seventh day of age the animals were put on limited feeding, they were allowed to eat for fifteen minutes. On each of the following two days they were given two preliminary trials which consisted of running from the entrance box to the goal box. The entrance and goal boxes were connected by a short straight pathway. After each trial

the rats were allowed to feed for one minute in the goal box and after both trials were permitted to feed for thirteen minutes in the feeding cage. On the sixtieth day of age they were given a preliminary trial in the complete maze.

From this point on each animal was given fifty trials in the maze two per day according to the following routine:

- 1 Taken from living cage and weighed
- 2 First trial in maze Permitted a nibble in the goal box
- 3 Second trial in maze Another nibble in the goal box
- 4 Fifteen minutes in the feeding cage
- 5 Returned to living cage

The running was started at the same time each day and the animals were run in the same succession. If the animal had not reached the goal box at the end of five minutes it was taken out of the maze and allowed to feed in the goal box for one minute then started on the second trial or placed in the feeding cage.

TABLE 7

*Group means and standard deviations of the two criteria of maze learning*

GROUP	NO. OF RATS	ERRORS		TIME (SECONDS)	
		Mean	S.D.	Mean	S.D.
Control	30	172.5	57.78	896.59	387.30
40	33	190.1	87.07	825.76	670.57
'80	30	199.9	70.80	878.48	628.00
120'	34	274.0	148.79	1314.03	1226.53

TABLE 8

*Comparison of the mean errors made on maze learning*

GROUPS COMPARED	DIFFERENCE BETWEEN MEANS	S.D.-diff.	DIFF. S.D.-diff.	CHANCES IN 100
120' — '80'	74.1	28.60	2.59	99.5
120' — 40	83.8	29.68	2.83	99.8
120 — Control	101.5	27.61	3.68	100
'80 — 40	9.8	19.67	0.50	66
'80 — Control	27.4	16.68	1.64	95
40 — Control	17.6	18.47	0.95	83

Two criteria of learning were employed: errors and time. Time from entering the maze to entering the goal box was recorded in seconds. An error consisted of (1) entrance into a cul-de-sac and (2) retracing the true pathway (each unit entered counted as a separate error).

**Results** The criterion of errors shows a positive relationship between the number of errors and the strength of the dosage: table 7 and fig. 3. The 120 group made a significantly greater number of errors than each of the other groups: table 8. The performance of the '80 group is probably reliably worse than that of the control group. The other differences are not significant.

The criterion of time shows that the 120' group is virtually significantly slower than each of the other groups, but the other groups do not differ among themselves: table 9, fig. 4.

TABLE 9  
Comparison of the mean time (seconds) for maze learning

GROUPS COMPARED	DIFFERENCE BETWEEN MEANS	S.D.-diff	$\frac{\text{DIFF}}{\text{S.D.-diff}}$	CHANCES IN 100
"120"—"80"	435.55	239.57	1.82	96
"120"—"40"	488.27	240.57	2.03	98
"120"—Control	417.44	221.92	1.88	97
"80"—"40"	52.72	163.62	0.32	63
"80"—Control	-18.11	134.71	0.13	55
"40"—Control	-70.83	136.48	0.52	70

TABLE 10  
Comparison of variability on maze learning  
Criterion = Errors

GROUPS COMPARED	DIFFERENCE BETWEEN S.D.'s	S.D.-diff	$\frac{\text{DIFF}}{\text{S.D.-diff}}$	CHANCES IN 100
"120"—"80"	77.99	20.22	3.86	100
"120"—"40"	61.72	20.98	2.94	99.8
"120"—Control	91.01	19.52	4.66	100
"80"—"40"	16.27	14.09	1.15	87
"80"—Control	13.02	11.80	1.10	86
"40"—Control	29.29	13.06	2.24	98.7

TABLE 11  
Comparison of Variability on maze learning  
Criterion = Time

GROUPS COMPARED	DIFFERENCE BETWEEN S.D.'s	S.D.-diff.	$\frac{\text{DIFF}}{\text{S.D.-diff}}$	CHANCES IN 100
"120"—"80"	598.53	169.32	3.53	100
"120"—"40"	555.96	170.07	3.27	100
"120"—Control	839.23	166.84	5.35	100
"80"—"40"	-42.57	115.09	0.37	64
"80"—Control	240.70	95.20	2.53	99.4
"40"—Control	283.27	96.52	2.93	99.8

TABLE 12  
Reliability of the maze based on errors per trial—split-half method

GROUP	r (SPEARMAN-BROWN)
Control	0.668 ± 0.109
"40"	0.972 ± 0.007
"80"	0.920 ± 0.021
"120"	0.985 ± 0.003

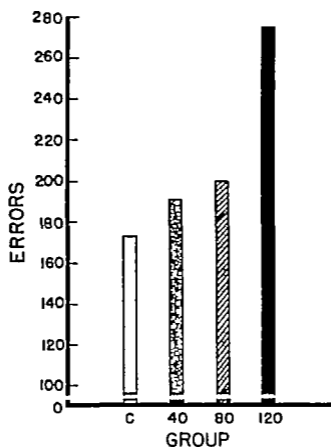


FIG 3 MEAN ERRORS PER GROUP ON MAZE LEARNING

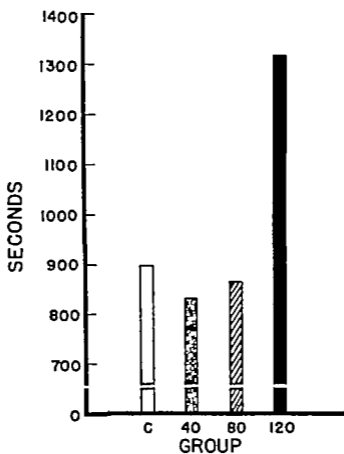


FIG 4 MEAN TIME PER GROUP ON MAZE LEARNING

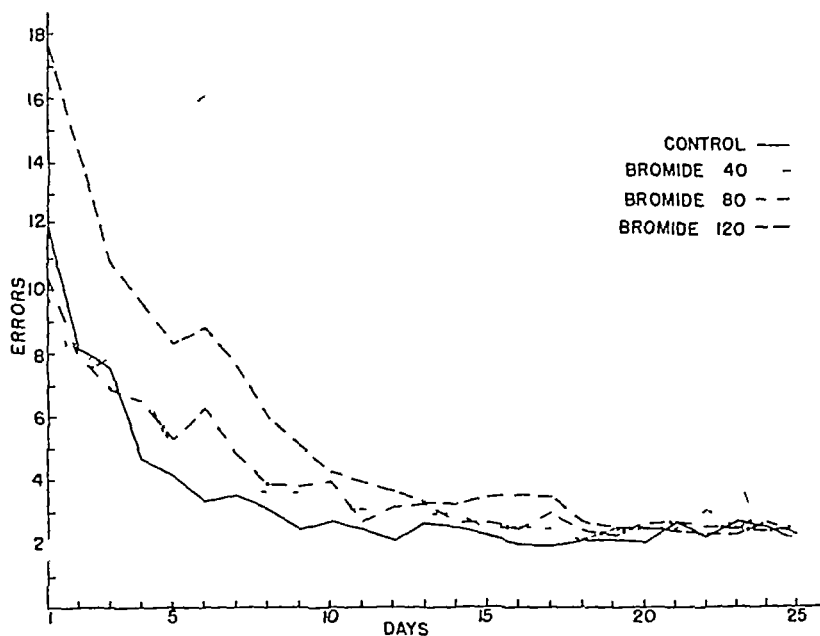


FIG 5 MEAN ERRORS PER TRIAL PER DAY

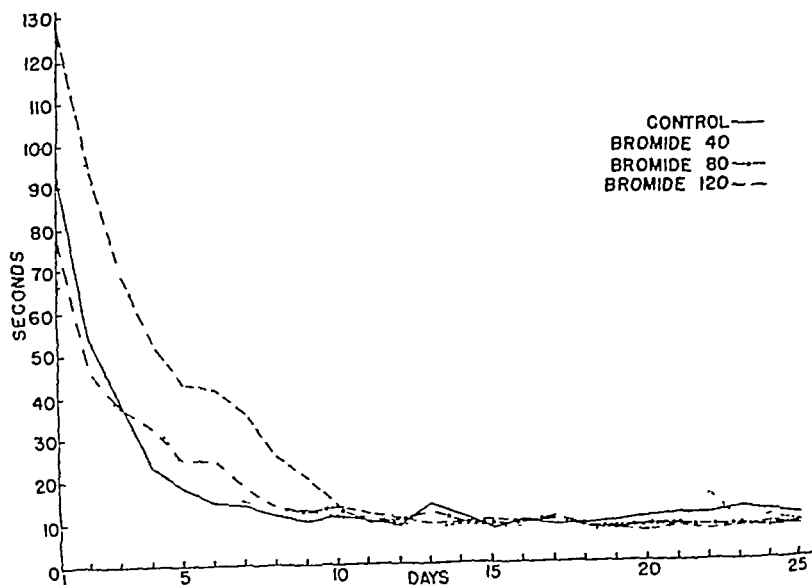


FIG 6 MEAN TIME PER TRIAL PER DAY

The error and time curves, figs. 5 and 6 show that all groups reached essentially the same level of performance before the end of the run (the 50th trial on the 25th day) suggesting that the deleterious effects of the factor being studied appear in the rate of learning rather than in the performance finally reached at least in this test

The standard deviations of the groups, computed on errors, show in general an increase with increasing dosage, table 7 Group 120" is significantly more variable than each of the other groups and group 40 is significantly more so than the control group table 10

The standard deviations computed on the time scores show the same relationships as those for error scores table 7, moreover, all groups differ significantly from one another, with the exception of the 80' and the 40 table 11

Reliability coefficients computed by the split-half method (odd-even days) are high three of the four being higher than those usually reported for mazes, table 12

**DISCUSSION** Studies similar to ours have been published by Maurer (9, 10), who reported that rats suckled during the first 12 to 15 days of life by mothers deficient in thiamine or riboflavin were definitely subnormal by the maze-test. Lashley (11 12) and Lashley and Wiley (13) have shown that there is a relationship between the amount of intact cerebral area and maze learning They reported correlation coefficients from 0.30 to 0.86 most of them from 0.80 to 0.86 between the size of the cerebral lesion and the errors in maze-tests Maier (14) also reported a high degree of relationship between the magnitude of the cerebral lesions and errors in maze learning A common denominator for Maurer's (9 10) results with vitamin deficiencies and our data with sodium bromide would appear to be provided by the assumption that these factors interfered with the development of the central nervous system The persistence of this effect is important and probably could have been determined by a repetition of the maze-test at an older age However, Maier (14) has shown that there is a significant correlation between the extent of cerebral lesions and the scores with his 8-table test for reasoning, hence, this test applied to our bromide-offspring some weeks after the maze-test would serve as a criterion of the persistence of functional damage and at the same time provide an additional measure of the injury Our results on this phase of the problem will appear in a forthcoming article (15)

# SUMMARY

1 Experiments were designed to test the hypothesis that sodium bromide administered during pregnancy would exert deleterious effects on the development of the nervous system of the offspring One hundred and twenty-seven rats of the Wistar experimental strain were used They were divided into 4 balanced groups the control, composed of offspring of normal mothers and three experimental groups whose mothers were dosed with 40 80 and 120 mgm of sodium bromide per day from the third through the twentieth day of gestation The animals were tested on a 5 unit U-maze from 61-85 days of age and at this time were free of abnormal amounts of bromide

2 The criterion of errors shows a positive relationship between the number of errors and the strength of the bromide-dosage

3 The criterion of time shows that group "120" which received the largest dose of bromide was significantly slower than each of the other groups but the other groups did not differ among themselves

4 All groups reached the same level of performance before the twenty-fifth day of the test This fact suggested that in the maze-test the deleterious effects of the bromide appear in the rate of learning rather than in the performance finally attained

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## A QUANTITATIVE STUDY OF THE EFFECTS OF OUABAIN UPON THE ELECTROCARDIOGRAM

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The purpose of the study herein reported was to determine, by the use of a truly quantitative approach, the effect of a cardiac glucoside upon the electrocardiograms (ECG's) of persons without heart disease. Ouabain was chosen because of its rapid and evanescent effect. In general the study consisted of careful comparison of control ECG's with others recorded at intervals after the intravenous administration of different doses of ouabain. It differs from other studies of similar purpose principally in that in the evaluation of changes in the T waves, the form of the preceding QRS complexes has not been ignored. Accurate evaluation of T wave changes necessarily involves determination of the ventricular gradient of Wilson (1).

**THE VENTRICULAR GRADIENT** The ventricular gradient is the electrical effect, as projected onto the frontal plane, of differences in the rate of repolarization of different regions of the ventricular muscle. If the rate of repolarization of all muscle elements were the same there would be no gradient, and the T waves would be opposite to the QRS complexes in direction and equal in net area in all leads. This statement neglects a possible effect of heart movement in systole. Under normal circumstances the rates of repolarization of different muscle elements are not the same, and the algebraic sum of the net QRS and the net T areas (QRS-T) in any lead constitutes a measurement of the electrical effect due to these differences in the rate of repolarization as projected onto the line of the lead. If measurement of the net area of QRS-T be made in any two of the limb leads and the Einthoven triangle method employed the direction as well as the manifest magnitude of the effect as projected onto the frontal plane may be determined. The vector thus determined, possessing both magnitude and direction is the ventricular gradient ( $G$ ). It should be mentioned that  $G$  is the frontal projection of the spatial gradient  $SG$  a vector in three dimensions, which is not accurately determinable by present methods (2).

The effect of digitalis glucosides upon the T wave is due to reduction of gradient magnitude that is to diminution or almost complete abolition of the differences in the rates of repolarization of different muscle elements. It causes the time course of repolarization of different muscle elements to become more nearly equal. Determination of  $G$  before and after digitalis administration should therefore furnish a truly quantitative study of this aspect of digitalis action providing no change occurs in the direction of the spatial gradient  $SG$ .

Figure 1 from cases of the present study demonstrates that an attempt to determine quantitatively by empirical observation the effect of digitalis upon the ECG would have been wholly unsuccessful.

Figure 1, A and B, is from patient J C, A before, and B thirty minutes after the administration of 15 mgm of ouabain. Apparent T wave change is slight, almost unnoticeable, yet G, after correction for the change in heart rate, was reduced from 45.0 to 33.3 microvolt seconds, a decrease of 26 per cent.

Figure 1, C and D, shows records from patient M K before and after the same dose of ouabain. T wave change is conspicuous, yet the corrected G was reduced very little more than in the preceding case from 32.4 to 23.0 m.v.s.,

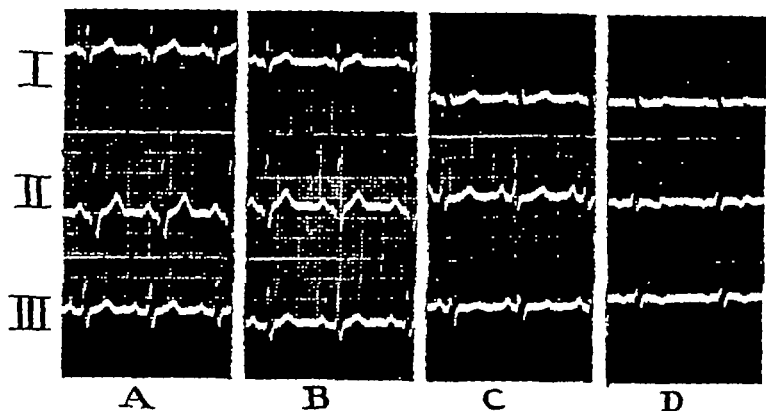


FIG. 1. A and B, from patient A C, were taken, respectively, before and one-half hour after the administration of the 15 mgm dose of ouabain. C and D, from patient M K, are similar. A comparison of these two cases illustrates the fact that T-wave, or RS-T segment, changes may give a misleading impression of the quantitative effect of the drug. The original measurements, after correction for the magnitude of  $A_{QRS}$  and for heart rate, indicated a 26 per cent reduction in the magnitude of the gradient in patient A C and a 29 per cent reduction in M K. Upon remeasurement by two of us, these decreases were estimated to be 26 and 33 per cent, respectively. (A correction for imperfect standardization was also necessary for patient M K. The change in amplitude of  $S_1$  in this patient was partly due to this factor.) The shortening of the Q-T interval in patient A C appears to be greater than in patient M K. (The constant  $k$  is decreased from 0.408 to 0.365 in A C, and from 0.417 to 0.391 in M K.)<sup>1</sup>

<sup>1</sup> We realize that our figures will seem paradoxical to many readers, and a further word of discussion seems needed. In A and B of the figure the manifest QRS-T vector,  $\vec{G}$ , is nearly parallel to lead II. Hence, the net QRS-T area in lead II gives immediately very nearly the magnitude, G. We estimate that this area is decreased about 12 per cent from A to B. The slowing of the heart results in a correction of about another 10 per cent. The total decrease, after correction, therefore, is at least 20 per cent. In C and D of the figure, the mean manifest QRS-T vector is nearly parallel to lead I both before and after ouabain. Hence, nearly the full value of G is projected onto the line of lead I. The larger part of the QRS-T area in that lead is the QRS. After ouabain, the net QRS-T area is reduced over 20 per cent and the correction for rate adds a little over 10 per cent. The decrease in G is about 35%. But in this case, an actual decrease in the magnitude,  $A_{QRS}$ , makes a further correction necessary, leaving the net decrease at about 30%. This decrease in the G value, and also a moderate deviation of  $\vec{G}$  to the left, together account for the marked T-wave changes in leads II and III.

a change of 29 per cent. In general the greater the magnitude of QRS the greater is the effect upon T of a given percentage change in gradient magnitude.

**METHOD** *General.* Hospitalized patients not acutely ill and without signs of heart disease were given intravenously various amounts of ouabain. Electrocardiograms (the limb leads and  $CF_4$ ) were taken before and at intervals after the injections, and the following determinations made:

- (1) The mean minute heart rate
- (2) The P-R interval
- (3) The Q-T interval
- (4) The direction and magnitude of the mean manifest QRS complex ( $\bar{A}_{QMS}$ )
- (5) The magnitude and direction of the ventricular gradient (G)
- (6) The net area of the ventricular complex QRS-T in lead  $CF_4$ . Details of the methods of comparison are given in the description of results.

*Graded dosage (Method A).* To each of ten subjects successive doses of 0.5 mgm., 1 mgm. and 1.5 mgm. per 150 lbs. of body weight were given at weekly intervals and electrocardiograms taken at one-half hour, one hour and four, 24 and 48 hours following each injection. Two additional patients received only the 0.5 mgm. dose.

*Single large dose basal conditions (Method B).* To each of fourteen adults and four children under essentially basal conditions (rest in bed and no food for twelve hours or longer) a single dose of 1.5 mgm. per 150 lbs. of body weight was given and an ECG recorded 30 minutes later. This method was employed to eliminate as far as possible an effect of varying nervous or metabolic activity upon the gradient.

**RESULTS** *Chronology of Electrocardiographic Effect.* All effects definitely ascribable to the drug were maximal at one-half hour; there was slightly less effect at one hour, and definitely less at four hours. After 24 hours but little effect remained and all traces had disappeared 48 hours after the injection.

*Heart Rate.* Considerable variation in heart rate, often obviously unrelated to drug effect, was encountered, and in most individual cases there was no clear correlation between dosage and change in rate. Table 1, however, which compares the average rate of control electrocardiograms with those taken one-half hour and one hour after graded dosage of ouabain, shows an increasing diminution of average rate with increasing dosage and suggests that the drug actually affected the rate in some cases. This is not a new observation.

*P-R Interval.* With one exception in no case was there a change in the P-R interval which could be ascribed to a direct effect of ouabain. In the exceptional case (patient W. M.) in association with a slight increase in heart rate the P-R interval was prolonged from 0.17 to 0.28 sec. The gradient change in this subject was exceptionally large, the reduction being 44 per cent. Among the other 27 cases the greatest increase, amounting to 0.02 sec., was associated with a large decrease in the rate. In one case there was apparent shortening of the interval. These findings do not contradict the commonly accepted belief that large doses of digitalis may affect the P-R interval, for even following the largest doses employed in our experiment other ECG changes were usually much less pronounced than those frequently observed following therapeutic digitalization.

*Q-T Interval.* The effect was determined by comparison of the Q-T interval before and after administration of the drug with the calculated Q-T for the

heart rate according to the formula proposed by Ashman and Hull (3): This procedure makes allowance for change in Q-T due solely to change in heart rate. Other studies of change in the Q-T interval have been reported, notably that of Dieuaide, et al (4).

By this method of comparison, it was found that the interval was shortened by the 1.5 mgm dose in every case, although in a number of cases the decrease

TABLE 1  
*Mean minute heart rates*

	CONTROL	0.5 MUM	1.0 MUM.	1.5 MUM
10 patients	77.9	77.4	74.8	67.3
18 patients	82.4			73.4
7 children	81.8			77.9

TABLE 2  
*Average G magnitude in relation to  $A_{QTS}$  and heart rate*

MINUTE HEART RATE	MAGNITUDE $A_{QTS}$ IN 4 MICROVOLT-SEC. UNITS									
	1	2	3	4	5	6	7	8	9	10 and up
40	11.1	12.5	14.0	15.3	17.0	18.7	20.2	21.9	23.1	24.5
50	10.0	11.0	12.1	13.6	15.0	16.1	18.0	19.2	20.5	21.8
60	9.2	10.1	11.0	12.1	13.3	14.7	15.8	17.4	18.5	19.8
70	8.5	9.3	10.1	11.2	12.4	13.3	14.5	15.5	16.8	18.0
80	7.8	8.6	9.3	10.2	11.2	12.2	13.2	14.2	15.3	16.4
90	7.2	7.9	8.6	9.3	10.2	11.1	12.0	12.9	13.9	15.0
100	6.4	7.1	7.8	8.7	9.4	10.1	11.0	11.8	12.6	13.7
110	5.9	6.5	7.2	8.0	8.8	9.5	10.1	10.9	11.6	12.4
120	5.5	6.1	6.8	7.5	8.2	9.0	9.7	10.2	11.0	11.6
130	5.1	5.7	6.2	7.0	7.7	8.3	9.0	9.7	10.3	11.0
140	4.7	5.3	5.7	6.4	7.2	7.7	8.3	9.0	9.7	10.7
150	4.4	4.9	5.4	6.0	6.7	7.2	7.8	8.3	9.1	9.7
160	4.1	4.5	4.9	5.6	6.2	6.7	7.2	7.7	8.3	9.0
170	3.8	4.2	4.7	5.2	5.7	6.2	6.7	7.2	7.6	8.3
180	3.5	4.0	4.4	4.8	5.2	5.6	6.0	6.5	7.0	7.7
190	3.3	3.7	4.0	4.4	4.7	5.1	5.6	6.1	6.5	7.1

was only 0.01 sec. When, in the case of the ten subjects who were given graded doses, the mean effect of different dosage at intervals after injection were plotted, curves similar to those illustrating the decrease in G were obtained, which

<sup>1</sup> Example Patient C P

	RATE	ACTUAL Q-T	CALCULATED Q-T FOR HEART RATE	DEVIATION FROM CALCULATED Q-T
		SEC	SEC	SEC.
Control	85	0.35	0.343	+0.007
30 min after 1.5 mg ouabain	65	0.345	0.385	-0.040
"Net" change in Q-T				0.047 decrease

seemed to show that the average effect was proportional to dosage. The mean absolute shortening from the control values, at one-half hour, were 0.007 sec 0.014, and 0.034 seconds respectively, for the 0.5 mgm. 1 mgm., and 1.5 mgm doses. These differences in such a small number of cases cannot be regarded as very significant.

**QRS Complex** No QRS changes ascribable to the effect of the drug were observed. In five or six instances alterations occurred, attributable to change in heart rate to changes in intraventricular conduction, or to change in the intra thoracic position of the heart. Equal variations occurred in the control curves.

**Magnitude of the Ventricular Gradient.**  $G$  was estimated from the projection of  $\bar{A}_{QRS}$  onto leads I and III, with the value in lead II used to check the accuracy of the measurements. Details of the method have previously been published (2)

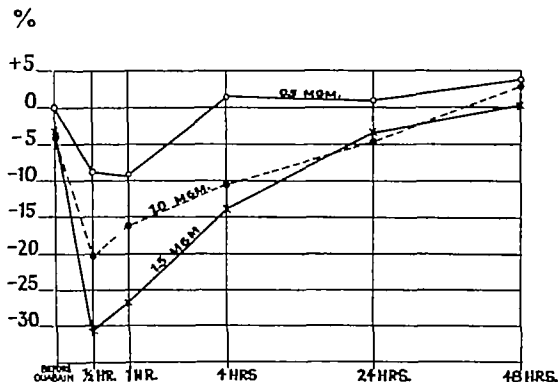


FIG 2

The magnitude,  $G$ , both in the control ECG's and in those recorded after ouabain administration were expressed in terms of percentages of the calculated  $G$  as obtained from table 2, and the change due to the drug is taken as the percentage difference between the two percentage values\*.

\* For example the measurements of patient S. R. may be tabulated as follows:

	HEART RATE	$\Lambda_{QRS}$	$G$	CALCULATED $G$ (TABLE 2)	$G$ , AS % OF ITS CALCULATED VALUE
		units			%
Control ECG	70	4.2	14.6	11.5	127
30 min after 1.5 mgm. dose	57	4.2	12.2	12.8	95

Percentage decrease in  $G$  apparently due to the drug: 25%

Note: Each unit equals 4 microvolt-seconds of area.

In figure 2 the mean G values, expressed as percentages of the absolute magnitudes after correction for heart rate, are shown for the ten subjects who received graded dosage according to method A. Progressively greater diminution of G with increasing dosage is demonstrated. At one-half hour, the mean decrease from the control magnitude was 8.5 per cent, 20 per cent, and 32 per cent, respectively, following 0.5, 1, and 1.5 mgm doses (table 3). The form of curve plotting effects of the largest dose may be roughly exponential, suggesting that the rate of elimination of the drug is proportional to the quantity remaining unaltered in the body at any given time.

Table 3 shows the percentage changes in G values in 12 cases, two of whom received only the 0.5 mgm dose. At one-half hour after injection, in terms of the *average* of the control magnitudes, namely, those before administration of the drug and those recorded 48 hours later, and after correction for heart rate, the effect of the 0.5 mgm dose varied from an apparent 18 per cent increase to a 32 per cent decrease in G in the different subjects. The 1.0 mgm dose produced changes in G ranging from an apparent 1 per cent increase to a 43 per cent decrease, and the 1.5 mgm dose produced apparent decreases ranging from 13 to 48 per cent. These subjects were not under nearly basal conditions. It is evident that the uncontrolled factors which may affect the gradient (and the T wave) may have a greater effect than the 0.5 mgm dose. In patient E. P., because of the small net areas, the percentage changes could not be accurately estimated.

Table 4 gives certain other data concerning the 10 of the patients of table 3 who received the 1.5 mgm dose. The meanings of the symbols are given in an earlier paper (8). In the last column are given the average percentage decreases in the gradient magnitude at one-half and one hour after the 1.5 dose. (Since these were recalculated from the original data by a somewhat different procedure, there are slight differences in a few cases between these figures and table 3.)

Table 4 is continued in table 5, but in the latter table, which included the 18 patients who received only the 1.5 mgm dose, the percentage decrease shown is not an average, but rather the decrease at one-half hour. In addition, this table shows the percentage decrease in the net QRS-T area in the C<sub>F</sub> lead, after correction for the left-leg potential and for rate. In table 5 the range of percentage decrease in G is from 5 to 46 per cent, but when the children are excluded, the range is from 22 to 46 per cent.

*Children and Adolescents vs. Adults.* Among the 28 subjects (excluding the two who received only the 0.5 mgm dose) used in this investigation there were 7 whose age ranged from 9 to 15 years, the other subjects ranged from 22 to 56 years of age. The range of percentage reduction of G produced by the 1.5 mgm dose per 150 lbs of body weight in the children was from 5 to 30 per cent, the mean being 15.4 per cent. In the adult group of 21 subjects, the range in the decrease produced by this dosage was from 22 to 49 per cent, the mean being 33.2 per cent. There can, therefore, be little doubt that, under the conditions of our experiments, ouabain, administered in doses proportional to

TABLE 3

*Patients whose electrocardiograms were taken at 0.5 1 4 24 and 48 hours after 0.5 1.0 and 1.5 mgm. of ouabain per 150 lbs. of body weight*

Percentage difference from the mean control values all the ECG's taken before injection and 48 hours after injection being taken as controls

PATIENT SEX AND AGE	MM.	BEFORE	½ HR.	1 HR.	4 HRS.	24 HRS.	48 HRS.
A. H. F 26	0.5	0	+4	+6	+13	+6	+17
	1.0	-26	-43	-84	-19	-11	+6
	1.5	-6	-48	-49	-16	-3	+7
C. Po. M 42	0.5	+23	-5	-7			0
	1.0	-6	-25	-19	-14	-12	-8
	1.5	+4	-29	-30	-8	-5	-14
E. P. M 35	0.5	-2	-4	-3	-1	+2	-16
	1.0	+15	+1	+1	+7	+5	0
	1.5	-14	-29	-14	-6	-1	+15
J. D. C. M 32	0.5	+15	-13	-10	-1	-2	+1
	1.0	-8	-27	-26	-19	+7	-4
	1.5	+3	-34	-39	-28	-4	-8
J. W. M 32	0.5	-15	-15	-9	0	+8	+11
	1.0	-8	-23	-23	-33	-20	+10
	1.5	+1	-43	-30	-3		
R. B. M 40	0.5	+16	-10	-8	-3	+4	-6
	1.0	-2	-13	-16	+1	-8	+6
	1.5	+2	-28	-29	-11	+1	-11
S. E. F 22	0.5	0	+18	0	+32	+4	+7
	1.0	-10	-24	-16	-14	+2	+9
	1.5	-12	-34	-32	-16	-12	+6
S. D. M (adult)	0.5	-12	-32	-24	-5	+11	+10
D. L. F 14	0.5	+4	-4	+3	+6	+5	+5
	1.0	+3		-17	-11	-12	
	1.5	-5	-13	-13	-16	-10	-8
G. W. M 13	0.5	-12	-10	-31	-12	-16	-3
	1.0	-1	-25	-7	-2	-1	-1
	1.5	0	-26	-16	+2	-5	+16
R. S. F 14	0.5	-16	-17	-11	+2	-2	+5
	1.0	+2	-6	-2	+2	+4	+8
	1.5	-2	-30	-29	-33	+2	-1
V. W. F 11	0.5	-5	-11	-18	-8	-3	+5

body weight, produces less electrocardiographic change in children than in adults

*Magnitude of QRS-T in Lead C<sub>4</sub>F* In the interpretation of ECG's taken in the C<sub>4</sub>F position, correction was made for left leg potential so as to make the results those of a unipolar lead (5) The measurements were made with care only on the later series of 18 subjects, and are more subject to error than the limb lead measurements

On the average in the adults, the net area of QRS-T in lead C<sub>4</sub>F was reduced by about the same percentage as the magnitude of the gradient The mean

TABLE 4

*Patients whose electrocardiograms were taken at 0.5, 1, 4, 24, and 48 hours after 0.5, 1.0, and 1.5 mgm ouabain per 150 lbs of body weight*

(The percentage decrease is the average of the change in  $\bar{G}$  at  $\frac{1}{2}$  and 1 hour after the 1.5 mgm dose only)

PATIENT	AGE AND SEX		MEAN HEART RATE	MEAN $\bar{A}_{QRS}$	MEAN $\bar{A}_{QRS}$	MEAN $\bar{G}$	MEAN $\bar{G}$	MEAN CALC. $\bar{G}$	PERCENT AGE DECREASE
A H	26 F	Before 1.5 mgm	88	+66°	4.0	+56°	6.7	9.5	
		After	65	+70°	4.1	+53°	4.2	11.7	48
C Po	42 M	Before	93	+39°	10.1	+41°	14.0	14.7	
		After	68	+38°	9.6	+35°	12.0	17.6	29
E P	35 M	Before	71	-44°	3.0	-15°	3.8	10.0	
		After	69	-46°	2.9	-51°	3.2	10.3	23
J D C	32 M	Before	64	+68°	8.6	+66°	16.0	17.4	
		After	62	+67°	8.2	+63°	10.1	17.2	36
J W	32 M	Before	53	+41°	4.4	+43°	12.1	14.1	
		After	48	+42°	4.2	+43°	7.8	14.3	37
R B	40 M	Before	70	+57°	7.2	+62°	14.8	14.8	
		After	65	+57°	6.8	+61°	10.7	14.9	28
S E	22 F	Before	80	+54°	6.3	+55°	11.4	12.6	
		After	78	+50°	5.5	+49°	7.2	11.9	33
D L	14 F	Before	92	+27°	6.2	+25°	12.5	11.1	
		After	80	+26°	6.0	+16°	12.0	12.2	13
G W	13 M	Before	90	+55°	3.8	+37°	12.6	9.2	
		After	81	+61°	3.8	+43°	10.7	9.9	21
R S	14 F	Before	94	+63°	6.1	+46°	13.8	10.9	
		After	83	+62°	5.6	+41°	9.2	11.2	32

decrease in 12 adult patients one-half hour after the 1.5 mgm dose was 38.5 per cent, the range being from 17 to 65 per cent The greater range may be ascribed mainly to the fact that the precordial ECG is influenced predominantly by local changes in the subjacent heart wall The average decrease in QRS-T, in children appears to be less than it is in adults

*Directional Changes of the Ventricular Gradient* This factor was determined at one-half hour after administration of the 1.5 mgm dose In 12 subjects no change in the direction of  $\bar{G}$  was apparent In seven subjects  $\bar{G}$  was clearly and in five slightly deviated to the left of its former direction In four cases,

TABLE 5

PATIENT	AGE AND SEX		HEART RATE	$\Delta QRS$ DEGREES	$\Delta QRS$ IN 4 M.V.S. UNITS	$\Delta$ DEGREES	G IN 4 M.V.S. UNITS	CALC. Q (TAB. 1)	PERCENTAGE INCREASE IN G	PERCENTAGE INCREASE IN QRS-T <sub>1</sub>
Adult patients who received the single dose of 1.5 mgm of ouabain per 150 lbs. of body weight										
A C	33 M	Before ouabain	97	+58°	3.15	+58	11.25	8.0		
		1/2 hr after ouabain	87	+58	3.1	+58.5*	9.1	8.7	26	17
C P	28 F	Before	85	+55	4.8	+41	7.1	10.6		
		After	64.5	+54	5.0	+34	5.8	12.8	34	23
D P	50 M	Before	80	+30	1.7	+31	1.9	8.3		
		After	67	+46	2.2	-6	1.5	9.6	35	65
E S	27 M	Before	85	+37	3.7	+35	7.9	9.5		
		After	60	+37*	3.7	+15	6.2	11.7	36	11*
F M	33 M	Before	83	-58°	3.2	+47	4.4	?		
		After	75	-55	3.3	+57	3.7	?	23(?)	48
H. R.	27 M	Before	77.5	-4	3.0	+14	6.3	9.5		
		After	77	-2	3.0	-43	3.5	9.5	44	
M K	34 F	Before	87.5	-11	5.9	+9	8.1	11.1		
		After	76	+8	5.5	+3	6.5	12.4	29	43
M M	35 M	Before	85	-1	3.7	+20	7.7	9.5		
		After	73.5	+12°	3.0	+20*	6.5	10.1	22	43
O B	34 M	Before	77	+76	4.3	+55*	8.1	11.3		
		After	70	+87°	3.65	+65	5.5	10.7	29	43
S M	28 F	Before	95	+54	6.5	+68	14.8	11.0		
		After	60.5	+56	6.8	+66	11.0	15.2	46	64
S R.	29 M	Before	70	+45	4.2	+48.5*	14.6	11.5		
		After	57	+45	4.2	+47	12.2	12.8	25	15*
W M	33 M	Before	87	+30	2.0	+30*	6.9	8.1		
		After	93	+40*	1.9	+13	5.0	7.6	44	53
W M T.	56 M	Before	82	+40	4.8	+31	3.8	10.6		
		After	82	+40*	4.8	+9	2.7	10.6	23	25
W R	26 M	Before	74.5	+63	9.4	+46	18.8	16.6		
		After	65	+64	9.0	+53	12.7	17.6	36	18

Children who received the single dose of 1.5 mg of ouabain per 150 lbs of body weight

C K.	11 F	Before	81.5	+45	3.9	+33.5	13.1	10.0		
		After	85	+45	3.9	+40	11.4	9.8	11	20
C S	11 F	Before	56	+81	10.4	+72*	20.1	21.2		
		After	60	+81	10.4	+72.5*	18.6	20.4	10	
R. L.	15 M	Before	80	+60*	3.6	+63	11.4	9.8		
		After	72	+60	3.6	+61.5	10.5	10.5	13	20
S C	11 F	Before	89	+30*	2.0	+15	6.2	8.0		
		After	86	+30*	2.0	-13	6.1	8.2	5	45

\*These values unreliable because of change in position of chest electrode

slight deviation of  $\bar{G}$  to the right occurred. It is interesting to note that the form of the QRS complexes indicated clockwise rotation of the heart about its longitudinal axis  $\bar{SH}$  (6, 7) in the cases showing rightward deviation, and either counterclockwise rotation, or no rotation in semihorizontal hearts, in all but one of the cases showing clear deviation of  $\bar{G}$  to the left.

A rather striking correlation was found to exist between the magnitude of the gradient in the control ECG's and the occurrence of leftward deviation of  $\bar{G}$  following ouabain administration. In eight subjects the magnitude of the gradient was less than 36 m v.s (90 units), and the largest leftward deviations occurred in the group, one of them showed no measurable deviation, and one was shifted  $10^\circ$  to the right. In patient O B, both  $\bar{A}_{QRS}$  and  $\bar{G}$  show changes apparently due to a change in heart position. In M M a similar anatomical change seems to have masked a leftward shift of  $\bar{G}$ .

These observations suggest the possibility that the reduction in  $G$  values may often be due, not only to a decrease in the magnitude of the spatial gradient,  $S\bar{G}$ , but also to a shift in the direction of the spatial gradient relative to the frontal plane, so that it has a greater backward or posterior direction (8). Our data do not permit us to decide this question. It is probable that this question must be studied more thoroughly before any serious attempt to use changes in the magnitude of the gradient for assay purposes is undertaken.

**Discussion** Insofar as T wave changes are concerned, this study is not comparable to those of other investigators, which consist of qualitative observations, despite the authors' opinions to the contrary. Moreover, whatever may be the conclusions of this study, they cannot definitely disprove the statement of Geiger, Blaney and Druckemiller (9) that "the electrocardiogram has no general clinical value as a quantitative measure of digitalization in the therapeutic range." Our studies have been limited to the effect of a single glucoside in clinically normal hearts, and we cannot predict the quantitative response in the several types of diseased hearts and in the presence of varied ECG abnormalities which exist prior to digitalization. Further, it is obvious that in the case of a single ECG of a patient to whom digitalis has been administered, the electrocardiographer has no way of judging the relative rôles of heart disease and digitalis effect in the production of observed abnormality of the gradient.

Our paper leaves unanswered the question as to whether or not the effect of ouabain upon the electromotive forces produced by the beating ventricles is quantitatively substantially the same in different normal hearts. It is apparently shown by our data that the effect is much less regular in children and adolescents than in adults. As we measure the changes, the range in percentage reduction in gradient magnitude brought about by the 15 mgm dose in adults was from 22 to 48 per cent. The consistency of the effects from person to person is, therefore, much greater than the variability in effects on the T waves would lead one to believe, and they may actually be much more consistent than these figures would suggest. As a measure of the actual change in the net electromotive force produced by the heart, a change which should be proportional to the change in the magnitude of the spatial gradient,  $S\bar{G}$ , our data are

affected by several unavoidable uncertainties and errors. In the first place, it is very likely that the weight of the patient is not an adequate guide to dosage. It seems more likely that the total volume of body fluid would serve as a better basis for regulating dosage so that the quantities administered to different individuals would be comparable pharmacodynamically. In the second place, errors occur in the measurements of the areas of the deflections and further errors in the correction for changes in heart rate. A third error is involved in the fact that we are measuring not the magnitude of the spatial gradient, but of that vector as it is projected onto the frontal plane of the body. If its direction were to remain unchanged then little or no error would be introduced, but, as we have seen, its direction may change, and the magnitude of this change may vary from person to person. Finally it is possible that the maximum change produced by the drug may sometimes have appeared before the lapse of 30 minutes after its administration.

It is probable that further study will make it possible to reduce all of these errors except that involved in the measurements, to a minimum and the latter could also be reduced by recording the curves on a more rapid film, with double timing even without resort to much more tedious procedures.

In view of the uncertainties it is impossible to express an opinion regarding the constancy of effect of ouabain upon the net electromotive force developed by the heart. One thing however is clear. The quantitative effects in different hearts are much less in normal persons, with ouabain and within the range of dosage employed, than qualitative observations could have suggested. If a large enough number of adult individuals are studied under controlled conditions quantitatively reliable mean effects are obtainable and these may be valuable in several lines of investigation including the assay of digitalis glucosides.

*Possibilities in the Assay of Digitalis Preparations* Although we have not attempted to utilize the method of estimating the effect of the glucoside on the magnitude of the gradient in biological assay of the drug in man the possibilities are evident. The only serious attempt of which we are aware to use T wave and RS-T electrocardiographic changes in assay on the normal heart is that of Gold, Cattell, Otto, Kwit and Kramer (10). They examined the RS-T segment changes, in leads I or/and II, in a series of 97 patients after administration of varying dosages of digitalis and in this number they found 18 who proved to be satisfactory subjects for the assay of digitalis. Each of these subjects was calibrated by giving successively three different doses of a U.S.P. Reference Digitalis Powder, differing from each other by 22 per cent. The unknown drug might then have been found to produce RS-T segment and T wave changes of nearly the same degree as one of the known doses or an effect intermediate between two of them thus giving five measurable stages of effect. By testing several of the calibrated subjects the potency of the unknown could be estimated with a reliability of 25 per cent or better. The method has the disadvantages that the test subjects must first be carefully selected from a larger number of individuals that several months are required for the calibration,

that the estimation of effect is not strictly quantitative but depends on the judgment of the electrocardiographer, and that no correction is made for rate fluctuations

After the 7 children and adolescents are excluded from our series, we find the mean decrease in the magnitude of the gradient produced by the 1.5 mgm dose in 21 adults to be  $33.2 \pm 1.1$  per cent and the standard deviation to be  $7.43 \pm 0.78$  per cent. The mean effects of the 0.5 mgm and 1.0 mgm dosages suggest that these should be fairly reliable if larger numbers of persons were tested. It, therefore, seems probable that, after the establishment of norms for various dosages of different digitalis preparations on sufficiently large series of adult subjects, the potency of an unknown preparation may readily be found with a reliability of much better than 25 per cent on a series of not over 25 adult non-cardiac subjects selected almost at random, provided the dosage of the unknown which is administered is not too small. It is likely that further study, and the rejection of a small percentage of unsuitable electrocardiograms, would increase the reliability of the method. It is true that a carefully trained technician must be available to make the measurements, but in the studies of Gold, et al., the judgment of a trained electrocardiographer was required. When the apparatus for measurement of the ventricular gradient, reported by Johnston and Wilson, becomes available, the errors in measurement may be eliminated (11).

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# THE RENAL ELIMINATION OF SULFAMERAZINE, SULFAMETHAZINE SULFADIAZINE AND SULFATHIAZOLE BY THE DOG

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Renal functions determine the principal mode of both elimination and conservation of sulfonamides by the body. All the sulfonamides which exist in an ultrafilterable state in plasma are capable of being filtered from the blood stream at the glomerulus. Since filtration is normally nonselective for plasma constituents other than protein and the substances bound thereon it follows that the renal conservation of these compounds is a function of tubular reabsorption, a selective process.

The low order of solubility of sulfonamides present in high concentrations in urine during adequate therapy makes crystalluria potentially the greatest and clinically the most frequent source of complications. Recognition of this fact has prompted a number of suggestions for measures designed to safeguard the patient against the occurrence of renal complications. The most frequently recommended procedures for the minimizing of renal damage have been diuresis and alkalization of the urine by the ingestion of sodium bicarbonate sodium citrate etc. Both of these measures have been used clinically and experimentally (1) but little interest has been manifested in how the kidney handled sulfonamides under these altered conditions, the assumption being that the solubility of the compounds was the only factor affected thereby.

In spite of a lack of uniformity of results a number of studies have served to substantiate the view that diuresis or alkalization of the urine does influence the renal elimination of sulfonamides. Marshall Emerson and Cutting (2) concluded that in the dog increasing the rate of urine flow also increased the clearance of sulfanilamide. This conclusion was later substantiated by Stewart, Rourke and Allen (3) and questioned by Lucas and Mitchell (4). Bullock, Ratish, Davidson and Lebar (5) reported that in man the excretion of sulfapyridine was independent of urine flow and Loomis, Koepf and Hubbard found the same to be true for sulfanilamide (6). Recently Peterson Goodwin and Findland (7) studied the effect of high fluid intake and also the ingestion of sodium bicarbonate on the rate of excretion of sulfadiazine. While actual sulfadiazine clearances were not measured they concluded that sodium bicarbonate produced a greater and more sustained elevated rate of excretion of this compound than did the increase in fluid intake.

The purpose of this research was to obtain definite experimental evidence concerning the effect of the following factors on the renal clearance of sulfamerazine, sulfamethazine sulfadiazine and sulfathiazole 1) the ingestion of sodium bicarbonate 2) low and high urine flow 3) low and high sulfonamide blood

levels, and 4) the effect of sodium bicarbonate on the clearance of sulfamerazine at low and high plasma levels

**EXPERIMENTAL** The dog is a very good experimental animal for this work since it does not conjugate sulfonamides. Consequently, determinations of the renal elimination of the free amines are not complicated by the simultaneous clearance of the related conjugated form of the compound.

In the course of any single experiment two or three simultaneous creatinine and sulfonamide control clearances were obtained, and then the procedure was altered to study the effect of any one of the previously mentioned factors on the clearance of the sulfonamide. About one-half hour before clearance studies were begun, 0.5 gram of the sulfonamide being studied and 2 to 2.5 grams of creatinine were injected subcutaneously. Forty cubic centimeters of water per kilogram of body weight were administered by stomach tube immediately following the creatinine injections. After the control periods more water or 7 grams of sodium bicarbonate in solution were given by stomach tube or more sulfonamide was injected subcutaneously, depending on the purpose of the experiment. Following another half-hour period for equilibration, three or more clearances were obtained under the altered conditions. Sulfonamide determinations were made by the method of Bratton and Marshall (8). Plasma albumin and total proteins were determined by a micro-Kjeldahl method on a sample of blood obtained from the animals at the time of each experiment to correct for the amount of sulfonamide which was bound.

Experiments also were carried out in which clearances were determined at gradually rising plasma levels of sulfonamide to study the relation between plasma level and the reabsorption of these compounds.

The plasma sulfonamide levels present in the table are not corrected for binding but are expressed in the conventional form of milligrams of determinable unconjugated compound per 100 cc. of plasma. Values for the creatinine clearances are given since they serve as an index of glomerular filtration rate in the dog (9). The clearance values for the sulfonamide are corrected for the binding of the respective compounds at the plasma level and the concentration of plasma albumin obtaining under the conditions of the experiment. The corrections are necessary since only the unbound sulfonamide is capable of being filtered (10). We have found that binding is limited to the albumin fraction of plasma proteins (11), which was in agreement with the published work of Davis (12).

Three dogs were used for each of the determinations. In general the same animals were available for all the experiments, thus cancelling out in a measure the factor of weight or surface area of the animals in the quantitative comparison of the various compounds and procedures.

**RESULTS AND INTERPRETATION** Table 1 summarizes data obtained on the four compounds. Qualitatively the changes are the same for the several sulfonamides in the various experiments, consequently the results may be discussed collectively.

*Normal elimination of sulfonamides* by dogs varied somewhat from animal to animal as might be expected yet the differences between the clearances of the four sulfonamides were distinct. These differences were most apparent when one compared the data for all the compounds for a single animal, as was done for dog #32 and #84. In ascending order the clearances on the various compounds were sulfamethazine (7.1, av.), sulfamerazine (9.3, av.), sulfadiazine (15.8, av.), and sulfathiazole (35.4, av.).

The bindings of these compounds on plasma protein (albumin) at approximately the concentrations that obtain in these experiments were as follows (11)

TABLE 1

*Renal elimination as influenced by sodium bicarbonate (pH) plasma level and rate of urine flow*

SULFONAMIDE	BEFORE AFTER	PLASMA CONC. IN MOM./100 CC.	URINE VOLUME PER MINUTE	CREATI- NINE CLEAR- ANCE	SULFO- NAMIDE CLEAR- ANCE†	CLEAR- ANCE RATIO	EXCRETION RATIO	PERCENT- AGE REAB- SORBED	pH
Effect of NaHCO <sub>3</sub> (pH)									
Sulfamerazine	B	4.4	2.34	53.2	9.3	0.13	0.17	82	6.78
	A	4.5	5.45	53.6	22.8	0.35	0.45	55	7.61
	B‡	3.5	1.85	46.9	13.0	0.20	0.23	71	7.80
	A‡	9.6	2.44	48.3	19.5	0.30	0.41	63	8.20
Sulfamethazine	B	5.6	3.41	56.9	7.1	0.07	0.13	87	6.76
	A	6.2	6.08	54.1	12.8	0.13	0.24	75	7.93
Sulfadiazine	B	4.5	3.65	61.2	15.8	0.22	0.26	75	6.85
	A	4.6	4.84	57.9	33.5	0.49	0.58	42	7.71
Sulfathiazole	B	4.4	3.51	58.9	35.4	0.30	0.60	40	6.88
	A	4.4	3.68	53.0	54.1	0.51	1.02	5	7.91
Effect of low and high plasma levels									
Sulfamerazine	B	3.8	4.20	62.7	12.5	0.14	0.19	81	6.80
	A	10.4	4.25	56.0	11.5	0.16	0.21	79	6.80
Sulfamethazine	B	5.7	2.97	56.1	7.6	0.06	0.14	86	6.60
	A	9.5	3.34	53.1	7.8	0.06	0.15	87	6.78
Sulfadiazine	B	5.0	4.08	61.9	13.0	0.21	0.25	79	6.41
	A	13.8	4.49	58.7	12.3	0.24	0.27	80	6.49
Sulfathiazole	B	5.0	3.17	51.9	33.5	0.31	0.66	38	6.61
	A	11.5	2.66	51.0	35.6	0.33	0.62	29	6.67
Effect of low and high urine flow									
Sulfamerazine	B	4.3	0.33	48.8	6.2	0.09	0.13	87	6.77
	A	4.3	5.12	63.4	18.8	0.21	0.30	71	6.76
Sulfamethazine	B	5.6	0.41	51.8	7.1	0.07	0.15	83	6.80
	A	5.2	3.77	60.3	20.5	0.15	0.35	65	6.62
Sulfadiazine	B	5.8	0.32	49.8	8.4	0.14	0.16	84	6.97
	A	5.2	4.72	63.6	16.8	0.22	0.28	75	6.52
Sulfathiazole	B	4.5	0.30	54.8	29.9	0.23	0.49	48	6.5
	A	4.4	5.50	69.8	55.2	0.35	0.78	21	6.2

Before and after sodium bicarbonate change in plasma level and change in urine flow

† Corrected for binding

‡ Effect of NaHCO<sub>3</sub> at low and high plasma concentrations

Sulfadiazine 16.4 per cent at a plasma level of 5.3 mgm/100 cc Sulfamerazine 36.5 per cent at a plasma level of 6.0 mgm/100 cc Sulfathiazole 53.2 per cent at a plasma level of 6.2 mgm/100 cc Sulfamethazine 60.7 per cent at a plasma level of 6.0 mgm/100 cc

Sulfathiazole was reabsorbed to the least extent of any of the compounds (40 per cent, av.) Sulfadiazine, which was filtered to the greatest extent, was the least reabsorbed of any of the pyrimidines. The values for the reabsorption of these compounds were sulfadiazine (75 per cent, av.), sulfamerazine (82 per cent, av.) and sulfamethazine (87 per cent, av.). Our figures for the clearance and excretion ratio of sulfamerazine, sulfadiazine and sulfathiazole were in good agreement with those of Fisher, Troast, Waterhouse and Shannon (13). Earle (14) has found that in man the difference between clearances for sulfamerazine and sulfadiazine was much greater than we found to be true for the dog. The clearance figures were 3.9 cc/min for sulfamerazine and 11.6 to 33.6 cc/min for sulfadiazine.

The effect of alkali on the renal elimination of sulfonamides was to increase clearance by decreasing the tubular reabsorption of the compounds in every instance. The absolute values for the increase in excretion ratio (decrease in reabsorption) following the administration of alkali increased in the following order: sulfamethazine, sulfamerazine, sulfadiazine and sulfathiazole. The most striking effect of alkalization was the increase in the excretion ratio to about 1.0 in the case of sulfathiazole. This meant that when sodium bicarbonate was administered the reabsorption of sulfathiazole was abolished and the renal elimination of the compound became entirely a function of the filtration rate of the kidneys.

A plausible interpretation of the effect of sodium bicarbonate on the tubular reabsorption of sulfonamides is that the electrolyte competes with the sulfonamides for the same reabsorption mechanism, and that there exists a functional capacity for the tubular reabsorption of compounds which otherwise do not appear to be closely related. These data lead one to believe that there may be relative affinities expressed by the renal tubular epithelium for the reabsorption of sulfonamides and sodium bicarbonate so that the latter compound can effectively displace the former, hence preventing their reabsorption. Reasoning along these lines, the observations that the tubular reabsorption of sulfamethazine is somewhat greater and the effect of alkali on that reabsorption less than for the other pyrimidines are consistent with the conclusion that the reabsorptive affinity of the tubules for sulfamethazine was greatest.

The data presented herein do not permit divorcing the effects of pH change from those of increased electrolyte excretion *per se*. However, it has been shown by Earle (14) that the administration of ammonium chloride, sodium chloride and potassium chloride were capable, qualitatively, of inhibiting the reabsorption of sulfamerazine. Thus it appears that this phenomenon is fundamentally one of electrolyte competition with sulfonamide reabsorption.

The dogs were given sodium bicarbonate prior to a number of experiments where the clearance of sulfamerazine was determined at low and high plasma

levels. The clearances were above normal values at all blood levels. These were the only experiments in which the clearance of any of these compounds was consistently and considerably higher at the elevated sulfonamide plasma levels. This effect may possibly be accounted for by a progressive absorption of sodium bicarbonate from the gastrointestinal tract during the experiments.

*An effect of low and high plasma levels on the clearance of sulfonamides* might be thought to influence the elimination of these compounds. Actually, it was found that within the usual therapeutic range up to about 15 mgm./100 cc. of plasma the plasma concentration did not materially influence the clearance, excretion ratio or reabsorption of these agents. Data on these experiments also were included in the table.

Figure 1 was included to illustrate that as the rate of filtration of these compounds was increased by increasing the plasma concentration there was a

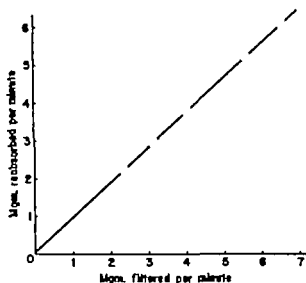


FIG. 1 DEMONSTRATING THE LINEAR RELATIONSHIP OF TUBULAR REABSORPTION TO GLOMERULAR FILTRATION OF SULFAMETHAZINE AT PLASMA CONCENTRATIONS OF THE COMPOUND UP TO APPROXIMATELY 15 Mgm./100 cc.

commensurate increase in the rate at which they were reabsorbed. The importance of this point was that if the functional ability of the renal tubules to reabsorb the compounds was exceeded, within the therapeutic range of plasma concentrations, much more sulfonamide would have remained in urine and the likelihood of renal complications due to obstruction would have been inordinately increased when the rate of glomerular filtration of the compound exceeded such a threshold value. Sulfamethazine was chosen simply for purposes of illustration. This generalization holds for the other compounds though some of our experiments permitted the feeling that the functional capacity of the tubules to reabsorb sulfathiazole was somewhat lower than for the pyrimidines.

*The effect of low and high urine flow on the renal elimination of sulfonamides* was studied using water to produce the diuresis. Consequently, we have thrown out several experiments in which the increase in urine flow did not exceed tenfold. There were only a few instances in which urine flow did not exceed 4 cc./min.

Increasing urine flow increased the renal clearance of all the sulfonamides tested. This inhibitory effect of diuresis on reabsorption was not so great as was the effect of sodium bicarbonate.

Experiments purporting to demonstrate the effect of diuresis on the clearance of sulfonamides were complicated by several factors which very definitely influenced the results. It should be noted that in many instances our experiments were begun at urine flows of about 0.2 cc/min. In most of these experiments both the creatinine and sulfonamide clearances were below their normal values for these dogs. Shannon (15) showed that during dehydration the creatinine clearance of dogs was low and concluded that this represented a decreased filtration rate during that state. It may be that this same explanation held for the decreased sulfonamide clearances at the low urine flows, yet an increased but undefined influence of passive back diffusion of the compound cannot be discarded as yet.

**DISCUSSION** These experiments serve to substantiate in part and also to modify the clinical concepts of the renal elimination of sulfonamides. The plasma protein binding of sulfonamides complements the renal tubular reabsorption of these compounds in the economy and maintenance of an adequate blood level of the unconjugated drug. From the standpoint of renal economy, then, it is advantageous that a compound be bound to a considerable extent in addition to being very markedly reabsorbed by the renal tubules. These two factors apply in the strict sense in which they are used here and yet they do not limit the modes of elimination of these compounds. If they were the limiting factors, sulfamethazine should have the greatest maintenance of blood level of the compounds studied. Actually, it is well known that the blood level of sulfamethazine is not sustained as well by single doses of the drug as is the case for sulfamerazine and sulfadiazine (14, 16). We have conducted further investigations in evaluating various factors in the elimination of these compounds which will be submitted as another communication.

These studies have shown that the use of sodium bicarbonate to increase the solubility of these compounds in urine was not entirely free from objectionable features. This agent depressed the tubular reabsorption of sulfonamides, and its effectiveness in this regard increased as the natural reabsorption of the compounds decreased. Thus, *sulfathiazole reabsorption was totally suppressed* whereas sulfamethazine reabsorption was least affected by alkalinization of the urine.

It is reasonable to anticipate that maintenance of a given sulfonamide blood level might be somewhat more difficult, requiring possibly larger or more frequent dosage in the presence of bicarbonate therapy. This has been shown to be true experimentally. Hughes and Schmidt (17) found when rats were fed a diet containing one or two per cent sulfamerazine or sulfadiazine the sulfonamide blood level was strikingly higher when sodium bicarbonate was omitted from the diet than when it, also, was fed. Acknowledging the above limitation on the usefulness of sodium bicarbonate one must still appreciate the usefulness of the drug in decreasing renal complications. The fact that it increases the solubility

of the sulfonamides is a point in favor but may not be the sole justification for its use in this connection. Hughes and Schmidt have accumulated evidence in the course of the experiments described above from which they concluded that sodium bicarbonate markedly reduced the renal pathology resulting from the administration of sulfadiazine in addition to diminishing the incidence of renal lithiasis when both sulfadiazine and sulfamerazine were fed to rats in high concentrations in their diets. Thus there is evidence for protection by sodium bicarbonate against a nephrotoxic action of sulfadiazine.

The results of our experiments permit the reassurance that as the concentration of these sulfonamides in the blood is maintained at high therapeutic levels, one has not added the additional hazard of having exceeded the maximal reabsorptive capacity of the renal tubules. Actually, as the plasma level of a compound is increased, and more of the drug is filtered there is an increase in the amount of the sulfonamide that is reabsorbed, hence the ratio between plasma concentration and the amount excreted per unit time (the clearance of the compound) remains fairly constant.

#### SUMMARY

The average renal clearance values, corrected for plasma binding for the four unconjugated sulfonamides were sulfamethazine (7.1, av.) sulfamerazine (9.3 av.), sulfadiazine (15.8, av.) and sulfathiazole (35.4 av.)

The administration of sodium bicarbonate increased the urinary pH but at the same time increased the clearances of all of the compounds by interfering with their reabsorption. This effect was most striking in the case of sulfathiazole where reabsorption of the compound by the renal tubules was almost completely inhibited.

The production of a water diuresis increased the clearance of each of the compounds but not quite to so great an extent as followed the administration of sodium bicarbonate.

As the plasma level of the sulfonamide was raised through the ordinary therapeutic range, there was a progressive increase in the tubular reabsorption of the compounds with the result that the clearance values did not tend to change with the plasma level.

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# THE NEUTRALIZATION OF GASTRIC ACIDITY WITH BASIC ALUMINUM AMINOACETATE

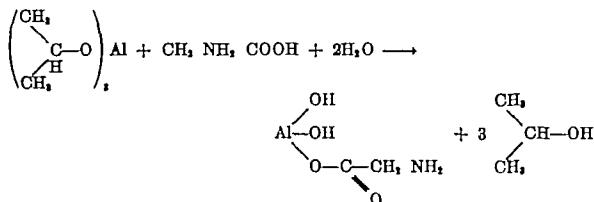
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For more than 15 years the amphoteric hydroxide of aluminum has played an important rôle in the treatment of gastric hyperacidity. As evidence of the recognition of the prevalent use and efficiency of this compound in the treatment of hyperacidity "Aluminum Hydroxide Gel" and "Dried Aluminum Hydroxide Gel" were admitted to the United States Pharmacopoeia XII. In the preparation of the descriptive monographs for the Pharmacopoeia the authors had an opportunity to observe the lag in acid adsorbing and neutralizing capacities of various samples of aluminum hydroxide gel. Accordingly it occurred to one of us that an aluminum salt of an aminoacid might give the dual effect of immediate acid neutralization by the amino group and secondary prolonged buffering of acid by the metathesis of the aluminum salt of the aminoacid and the strongly dissociated hydrochloric acid. In addition the resulting formation of aluminum chloride would exhibit astringency as is claimed when aluminum hydroxide is administered.

**CHEMICAL CONSIDERATIONS.** Efforts to prepare the aluminum salt of alanine and glycine from inorganic aluminum salts and the sodium salt of the respective aminoacid invariably resulted in the precipitation of aluminum hydroxide. One of us succeeded in preparing the compounds desired by the following reaction using aluminum isopropoxide (1)



A solution of freshly prepared aluminum isopropoxide (1 mole in anhydrous isopropyl alcohol) was added slowly to an aqueous solution of glycine (3 moles) heated on a steam bath. Since glycine is not readily soluble in any common solvent except water this could not be excluded from the reaction and hence the basic salt rather than the normal salt was formed. In our opinion under the conditions which we have found to be reproducible the isopropoxide in decomposing reacts with both water and glycine to yield aluminum dihydroxy-amino

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hydrochloric acid were placed in small Erlenmeyer flasks. To each volume respectively was added either dried aluminum hydroxide gel or basic aluminum glycine salt. The temperature was maintained at  $25 \pm 1^\circ\text{C}$ . After the addition of the varying molecular quantities of the respective antacids the mixture was thoroughly agitated. After exactly 5 minutes the pH was determined by means of a glass electrode. The results are set forth in chart 2.

It is apparent from the data in chart 2 that the action of the basic aluminum glycine salt is more prompt in its buffering of hydrochloric acid than is dried aluminum hydroxide gel.

**PROLONGED ACID-CONSUMING CAPACITY** It is well established that in addition to prompt relief of pain through acid neutralization and subsequent diminution

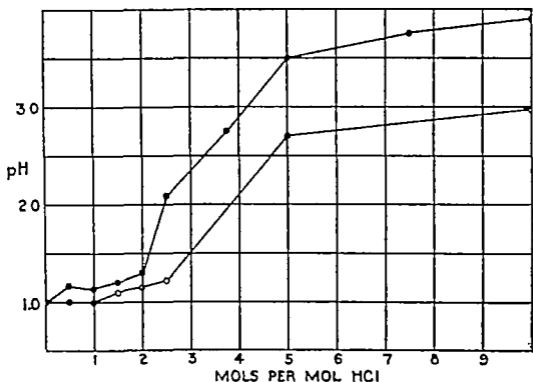


CHART No. 1

Dots Basic aluminum aminoacetate

Circles Basic aluminum aminoacetate plus excess of formaldehyde

of gastrointestinal motility prolonged buffering action is desirable in the treatment of peptic ulcer. Accordingly we studied the effect of basic aluminum aminoacetate on an artificial stomach contents used previously by one of us (3). The pH of the untreated gastric juice was 1.32. To 25 cc. portions of this juice the respective compounds were added under the conditions described previously. Hydrogen ion concentration determinations were carried out at  $\frac{1}{2}$  and 1 hour periods. The results are set forth in chart 3.

**BUFFER CAPACITY OF GLYCINE** The buffer value of the ampholyte, glycine was recognized by Sorensen who established a series of buffer mixtures using glycine, sodium chloride and hydrochloric acid. We prepared a tenth normal glycine solution and by the glass electrode found its pH to be  $6.40 \pm 0.05$ .

at 23°C The addition of an equal volume of tenth-normal hydrochloric acid, converting the ampholyte into its hydrochloride, changed the pH to 1.96. One and two-tenths, 1.4, 1.6 and 2.0 moles of hydrochloric acid to 1 mole of

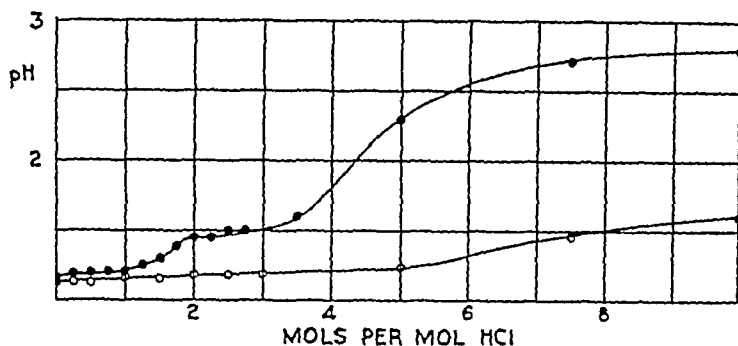


CHART No 2

Dots Basic aluminum aminoacetate  
Circles Dried aluminum hydroxide gel after 5 minutes

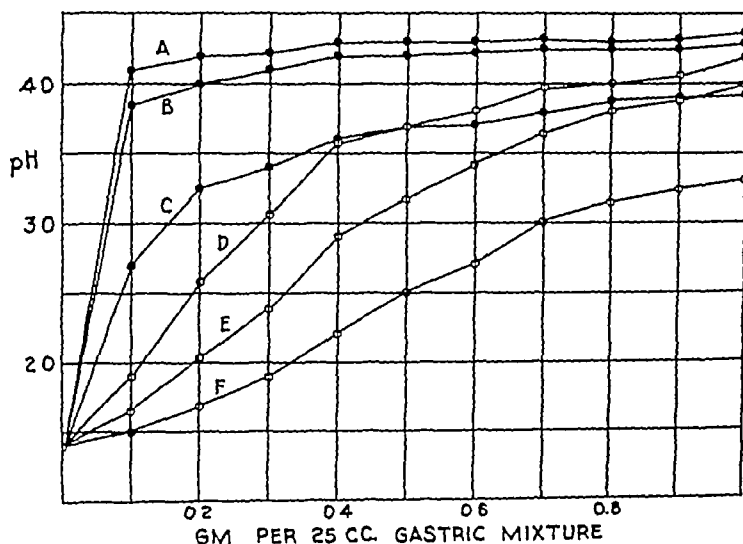


CHART No 3

Dots Basic aluminum aminoacetate, A = after 2 hrs, B = after 1 hr, C = after ½ hr  
Circles Dried aluminum hydroxide gel, D = after 2 hrs, E = after 1 hr, F = after ½ hr

aminoacetic acid changed the pH to 1.80, 1.68, 1.60 and 1.52 respectively. These values lie on the curve for the acid titration of glycine determined by Harris (4). The pH of tenth-normal hydrochloric acid is approximately 1, while that of the hydrochloride of glycine as shown by the foregoing values, is about 2,

thus the presence of glycine causes a ten fold diminution of the hydrogen ion concentration and the change is instantaneous

**BUFFER CAPACITY OF BASIC ALUMINUM AMINOACETATE.** Van Slyke (5) adopted the differential ratio  $\frac{dB}{d\text{pH}}$  to express the relationship between the increment (in gram equivalents per liter) of strong base 'B' added to a buffer mixture and the resulting increment of pH. The negative ratio may be used to express the buffer value upon the addition of strong acid. Accordingly a solution has a buffer value of one when a liter will take up one gram equivalent of strong acid per unit change in pH.

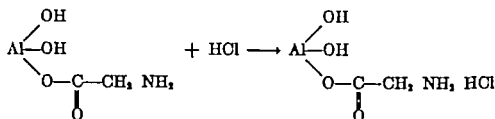
One of the characteristics of a useful gastric antacid is its capacity to buffer newly secreted acid from the oxyntic cells, by means of the residual neutralized and unneutralized antacid in the gastric contents. The following experiment was carried out. To 25 cc. volumes of artificial gastric contents were added 0.5 gm. respectively of basic aluminum aminoacetate and aluminum hydroxide gel. The mixtures were shaken intermittently for 2 hours permitting equilibrium to be established. Then a 5 cc. additional portion of the gastric mixture was added and after 5 minutes the pH was determined.

At equilibrium, the pH of the basic aluminum aminoacetate mixture was 4.20 after the addition of the acid the pH was changed to 4.09, a difference of 0.11. For dried aluminum hydroxide gel, the equilibrium pH was 3.73, after the addition of acid the pH changed to 3.12, a difference of 0.61. The 5 cc. of additional tenth normal hydrochloric acid added represents an increment of 0.02 moles per liter.

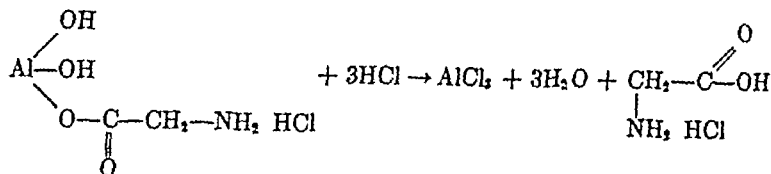
With gastric contents and dried aluminum hydroxide gel the approximate buffer capacity using the above measurable increments was calculated to be  $-\frac{\Delta B}{\Delta\text{pH}} = \frac{0.02}{0.61} = 0.033$  while with basic aluminum aminoacetate the value was  $\frac{0.02}{0.11} = 0.182$ . Therefore, the buffer capacity of basic aluminum aminoacetate is approximately six times that of dried aluminum hydroxide gel under these conditions.

The nature of the action of the amino acid derivative of aluminum is likely a function of its marked tendency toward colloidal dispersion, and of its ampholytic character. Amphoterics bodies, like proteins do not always pass into true solution and their behavior i.e., reactivity and pH are partially the result of interfacial equilibria of ionic micelles. In gastric contents, basic aluminum aminoacetate likely undergoes the following reactions

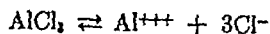
#### 1 Immediate neutralization



## 2 Delayed neutralization



## 3 Astringency



FEEDING EXPERIMENTS. A group of 20 rats (average weight 54 grams) was fed a supplemented Purina fox chow to which 2 per cent of the basic aluminum

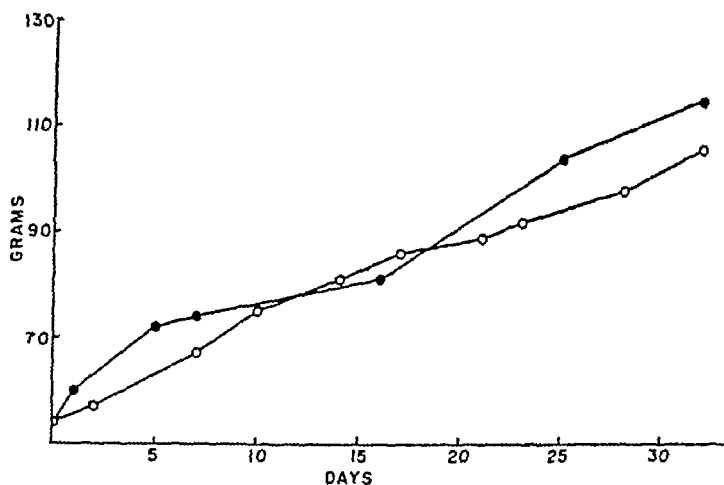


CHART No. 4 Growth curves of normal and basic aluminum aminoacetate-fed rats  
Circles Control

aminoacetate had been added. They were observed over a period of 4 weeks. The growth curves of these and control animals are shown in chart 4. At the termination of the experiment 3 animals were sacrificed and their livers, kidneys and a portion of the small intestine were examined histologically. There were no abnormalities observed.

INGESTION BY HUMANS. Basic aluminum aminoacetate was administered to 20 persons in doses of 2 to 4 gm in powder form, suspended in water. It was tolerated without symptoms. Two cases of active peptic ulcer were treated with the salt as the sole antacid. Healing was observed by the gastroscope and the patients became asymptomatic. Two cases of latent peptic ulcer, with post-prandial distention and hyperacidity obtained prompt and prolonged relief upon the ingestion of 0.5 gm compressed tablets of the salt. Extensive clinical

investigations with this compound are in progress. These will form the basis of a subsequent communication.

#### SUMMARY

1 A new compound, the basic aluminum salt of aminoacetic acid, has been prepared.

2 Its capacity to buffer and neutralize hydrochloric acid has been studied. Its prompt and prolonged buffering of acid, bespeaks the use of the compound in the treatment of hyperacidity and peptic ulcer. On the basis of the aluminum content, basic aluminum aminoacetate is 42 per cent more efficient in acid consuming power than dried aluminum hydroxide gel.

3 Certain theoretical considerations of its use have been discussed.

4. Feeding studies on rats and preliminary clinical trials as an antacid in man are recorded

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# PROTECTION AGAINST LETHAL DOSES OF PENTAVALENT ARSENICAL AND ANTIMONIAL COMPOUNDS IN RATS BY p-AMINOBENZOIC ACID, A HISTOLOGICAL STUDY

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**INTRODUCTION** It has been reported recently by Sandground (1, 2), and Sandground and Hamilton (3, 4) that p aminobenzoic acid not only does not inhibit the trypanocidal action of carbarsone and other organic pentavalent arsenical compounds, but that it actually affords protection to rats against lethal doses of such familiar phenyl arsonates as "Tryparsamide," arsamic acid, carbarsone, and acetarsone. Virtually the same results were obtained with "Stibosan," sodium m-chloro-p-acetylaminophenyl stibonate, a German proprietary pentavalent antimonial compound (5).

Although adequate studies of the toxicity of new arsenical compounds have been made, there have been few investigations of the pathology of poisoning in experimental animals by various arsenicals. In the case of "Tryparsamide" only two such publications have come to our attention. Brown and Pearce (6) administered "Tryparsamide" to rats, mice, guinea pigs, rabbits, and monkeys, and found the kidneys to be the site of the chief injury. They also observed a few scattered petechiae, occasional effusions into the serous cavities, and degeneration or necrosis of the adrenal glands and cardiovascular system. Animals which survived the acute intoxication rarely showed lesions in organs other than the kidneys and heart. The renal lesion produced in rats by "Tryparsamide" was described in detail by Bunting and Longley (7), who administered doses ranging from 1000 to 3000 mg per kg. They observed that with small doses the secondary convoluted tubules underwent necrosis, and that with large doses necrosis was evident earlier and involved the primary convoluted tubules as well. Evidence of glomerular injury was slight, and the collecting tubules were never injured. It is the purpose of this communication to discuss the protective action in rats of p aminobenzoic acid against toxic doses of "Tryparsamide" and "Stibosan" as revealed by microscopic examination of the viscera.

**METHODS** Both "Tryparsamide" and "Stibosan" were administered intravenously in doses which previously had been found to kill a high proportion of the animals. Half the rats also received p aminobenzoic acid orally or intraperitoneally at a level of 750 mg or 1 gm per kg on successive days over a period of one to five days, the first dose being given shortly before the arsenical or antimonial compound. These animals were obtained from Sandground early in his studies in the detoxication of organic arsenical compounds by p aminobenzoic acid, at which time he thought that repeated doses of p aminobenzoic acid might provide more complete protection than would single doses. Subsequently he found this surmise to be erroneous (4). The animals included in this study

constitute only a small proportion of the number used by Sandground in his investigations, for some rats which died had undergone too much post mortem change for satisfactory microscopic examination when found and not all survivors were killed. Only living or freshly dead animals were accepted for histological examination. For brevity and convenience we shall refer to the rats which received p-aminobenzoic acid as PABA rats and to the others as controls.

Within less than twenty-four hours the contrast between the condition of PABA and that of control animals was striking and became even more impressive during the second day. The control animals were usually lethargic while unmolested but on stimulation proved to be hyperirritable with tremors, ataxia, gyration and weakness. About their eyes there was usually a rim of red exudate a coproporphyrin (8), and often the eyelids were sealed together by this exudate. As the more severely injured rats were eliminated by death and the less injured ones began to recover it was still easy to distinguish between the PABA and control animals because of the better state of nutrition of the former. At the beginning of the experiment the rats were of the same size and age weighing about 90 or 100 grams. Very few PABA animals showed severe injury, and few died spontaneously. The majority showed only a slight and transitory indisposition. While not all control animals showed severe injury the majority did, and their condition steadily grew worse until death supervened. Whenever deaths occurred among the control group, one or more PABA rats was killed with chloroform for comparative histological examination.

In a study of the toxicity of p-aminobenzoic acid, Scott and Robbins (9) found rats to be very resistant, no deaths occurring following administration of single oral doses ranging up to six gm per kg and no visceral lesions being demonstrable microscopically in rats which had received daily doses of 1.4 grams per kg over a period of four weeks. In view of these results we considered superfluous the running of a control with administration of p-aminobenzoic acid alone.

**TRYPARSAMIDE POISONING** All animals in this group received a single dose of 3200 mg. per kg of Tryparsamide intravenously an LD<sub>50</sub> according to Sandground's data (2). The tissues of eleven control and ten PABA rats were examined microscopically. None of the PABA animals in this group died spontaneously. Control rats died on the following days after injection of Tryparsamide: one on the second, three on the third, two on the fourth, three on the fifth, and one on the sixth. One of the survivors was killed on the eleventh day. PABA rats were killed on the following days after administration of Tryparsamide: one on the second, two on the third, fourth and fifth, one on the sixth, and two on the eleventh. The rat which was killed on the second day received two daily doses of one gm per kg. of p-aminobenzoic acid. The remaining rats received daily doses of 750 mg or 1000 mg per kg for the first three days and no treatment thereafter.

Renal injury was the most conspicuous and consistently present lesion observed. Other lesions which were found affected only the control rats and included pulmonary edema in three rats, thymic atrophy in seven, focal myocardial necrosis in five, slight bleeding into the stomach or small intestines in

five, emaciation in two, hydrops of the liver in one, fatty metamorphosis of the liver in one, and atrophy of the liver in one. The renal lesions consisted in necrosis of convoluted tubules, the glomeruli and collecting tubules being uninvolved. Identification of completely necrotic tubules was often difficult or even impossible. Where necrosis was slight, it was apparent that the secondary convoluted tubules were the ones affected, but in the more severely injured kidneys the primary convoluted tubules and loops of Henle were also necrotic. In most tubules the necrotic cells were of normal size and had regular inner margins, nuclei had completely disappeared, and the cytoplasm was hyaline, eosinophilic, and homogeneous. The lumina of most tubules were empty, and there was no leukocytic infiltration. The necrotic cells of some tubules were swollen, and some had apparently lost some cytoplasm. Other tubules contained debris of necrotic cells. In some tubules necrosis was not complete, nuclei covered by a thin layer of viable cytoplasm lying against the basement membrane and beneath a thick layer of necrotic cytoplasm. The cells lining viable tubules were often swollen. Regeneration of tubules was active in some kidneys and not seen in others. It was manifested by basophilia of the cytoplasm, abundant mitoses, increased numbers of nuclei, and hence presumably increased numbers of cells, for cell boundaries were rarely distinguishable. Not infrequently the nuclei of regenerated cells were appreciably larger than normal.

The contrast between the kidneys of control and PABA rats was striking. Much necrosis of convoluted tubules was seen in the kidneys of the control rat which died on the second day, whereas the kidneys of the PABA rat killed on the same day showed little necrosis, but many mitoses. The kidneys of the three control rats which died on the third day showed much necrosis with mitoses in the less severely injured tubules. The kidneys of the two PABA rats which were killed on the same day were normal. The kidneys of the two control rats which died on the fourth day were similar to those of the rats which died a day earlier. The kidneys of one PABA rat killed on this day were normal, but the kidneys of the other showed slight regeneration with no necrosis. There was moderate necrosis of convoluted tubules in the three control rats which died on the fifth day, one showed much, a second slight, and the third no regeneration. The corresponding PABA animals were normal. The convoluted tubules of the control rat which died on the sixth day showed only slight necrosis and much regeneration. The kidneys of the corresponding PABA rat were normal, as were those of PABA and control rats killed on the eleventh day.

Insofar as the experiments are comparable, our observations are essentially the same as those of Bunting and Longley (7). However, we did observe that wherever the secondary convoluted tubule in the vicinity of the macula densa was recognizable, it was normal. Whether the remainder of such tubules were also normal, or whether this portion of the tubules is less readily injured, we cannot say.

**"STIBOSAN" POISONING** The rats in this group received intravenous injections of "Stibosan" at dosages ranging from 250 to 375 mg per kg of body weight. A few received two injections of the drug at the rate of 250 mg per

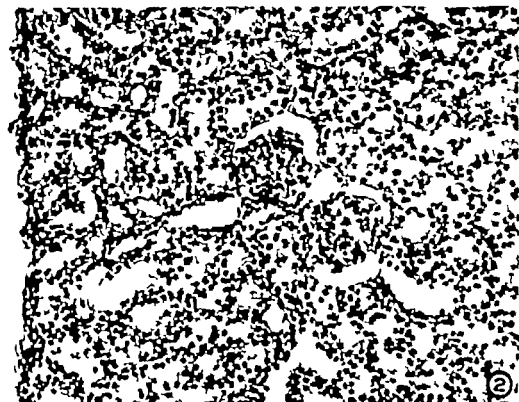
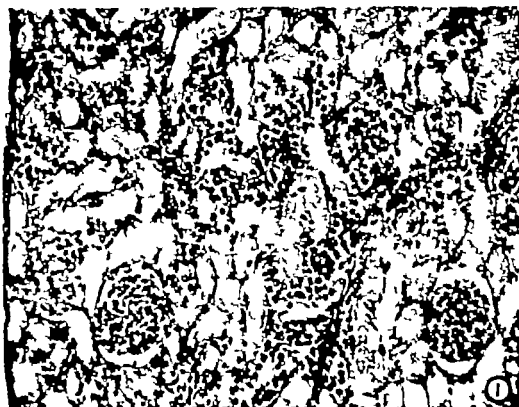


FIG. 1. 228X. KIDNEY OF RAT 22 WHICH RECEIVED 320 MG. PER KG. OF "TRYPAR-AMIDE" AND DIED ON THE FOURTH DAY.

The glomeruli and Bowman's capsule are essentially normal, while the tubules are completely necrotic.

FIG. 2. 228X. KIDNEY OF RAT 23 WHICH RECEIVED 320 MG. PER KG. OF "TRYPAR-AMIDE" AND ON THREE SUCCESSIVE DAYS 50 MG. PER KG. OF  $\beta$ -AMINOBENZOIC ACID.

It was killed on the fourth day. The tubules are normal in appearance.

kg on successive days. The PABA rats received daily injections of p amino benzoic acid at the rate of one gm per kg over a period of two to five days. Thirty-two control and seventeen PABA rats were available for histological examination.

As was true with "Tryparsamide" poisoning, renal injury was the most serious lesion present, and was similar in appearance and distribution to that produced by the arsenical compound, consisting in necrosis of the secondary convoluted tubules, but with involvement of the primary tubules occurring only rarely. Collecting tubules and glomeruli were not injured. Other lesions observed included focal myocardial necrosis in three control and one PABA rats, hydrops of the liver in five control and two PABA animals, fatty metamorphosis of the liver in four control and two PABA rats, hydrothorax in one control, pulmonary edema in eleven control and three PABA rats, atrophy of the thymus in nineteen control and five PABA animals, and an infarct of the adrenal gland in one control. Thymic atrophy and pulmonary edema were the only extra renal lesions which occurred with greater frequency in control rats than in PABA rats.

Two control animals died early on the second day of the experiment. The kidneys of both rats showed parenchymatous degeneration, and the kidneys of one also showed necrosis of some convoluted tubules. Later in the day two control and two PABA rats were killed. The kidneys of the former showed slight necrosis of convoluted tubules with some mitoses, whereas the kidneys of one of the latter showed slight necrosis of convoluted tubules and many mitoses, and the kidneys of the other were normal. On the third day one PABA and six control rats died, the kidneys of all showed slight necrosis of convoluted tubules with some mitoses and apparent increase in number of cells. On the fourth day seven controls died and four were killed, and two PABA rats died and four were killed. The kidneys of all but three of the controls showed slight necrosis, and all eleven showed much regeneration, with few to many mitoses. The kidneys of three PABA rats were normal, those of two showed slight regeneration and no necrosis, and the kidneys of one showed slight necrosis but no regeneration. On the fifth day one control rat died and four were killed, and three PABA rats were killed. The kidneys of one control and one PABA rat showed slight necrosis, mitoses and other evidences of regeneration were seen in the kidneys of all eight rats. On the seventh day one control rat died. Its kidneys showed regeneration without necrosis or mitoses. On the eighth day two control and two PABA rats were killed. The kidneys of the former showed regeneration with many mitoses and no necrosis, the kidneys of one of the latter were normal, and the kidneys of the other showed slight regeneration, but no mitoses or necrosis. On the tenth day one control and one PABA rat were killed. The kidneys of the control showed regeneration with no mitoses and no necrosis, and the kidneys of the latter were normal. On the eleventh day two rats from each group were killed. The kidneys of the controls and of one PABA rat showed some regeneration, but no mitoses or necrosis, while the kidneys of the other PABA rat were normal.

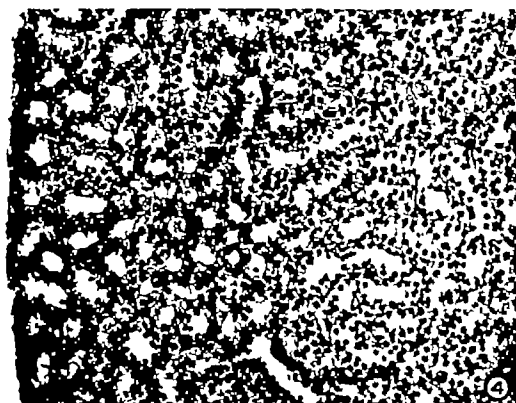
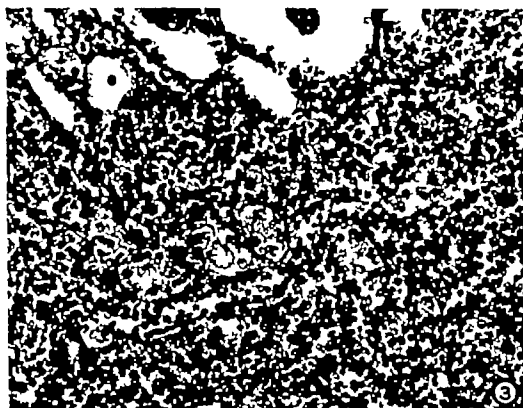


FIG. 3 228X KIDNEY OF RAT 11 WHICH RECEIVED 350 MG PER KG OF STIBONAN AND WAS KILLED ON THE FIFTH DAY.

Hypertrophy and increase in number of nuclei is apparent. A few tubules contain fragments of necrotic cells while others are dilated and empty.

FIG. 4 228X KIDNEY OF RAT 115 WHICH RECEIVED 350 MG PER KG OF STIBONAN AND 1 CM PER KG OF p-AMINOBENZYLIC ACID.

On the second day an additional dose of 500 mg per kg of p-aminobenzilic acid was given. The rat was killed on the fifth day. The condensed tubules appear normal.

**DISCUSSION** The phenomenon of protection by p aminobenzoic acid against poisoning by pentavalent arsenical and antimonial compounds is shown not only by a much lower mortality rate among the PABA rats, but by the much smaller incidence and extent of renal injury observed histologically. It is of great significance that of ten rats which received "Tryparsamide" and p aminobenzoic acid, the kidneys of eight were histologically normal, while the kidneys of only one of eleven which received "Tryparsamide" alone were normal, and that of seventeen rats which received "Stibosan" and p aminobenzoic acid, the kidneys of seven were histologically normal, and that of thirty two rats which received "Stibosan" alone the kidneys of none were entirely normal. Furthermore, the extent of regeneration was usually less among the PABA animals, a consequence of less severe and less extensive tubular injury. It seems improbable that p aminobenzoic acid caused an acceleration of regeneration with complete restoration to a normal status. From our results it appears that the protection offered against renal injury by "Tryparsamide" is more complete than that offered against injury by "Stibosan," but without more adequate data as to the toxicity of "Stibosan" we are unable to say unequivocally that the protection is greater.

#### SUMMARY

The protective action of p aminobenzoic acid against poisoning by "Tryparsamide" and "Stibosan" is manifested histologically by appreciable lessening of the extent and severity of renal damage produced by those compounds.

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# THE EFFECT OF ACID AND ALKALI ON THE ABSORPTION AND METABOLISM OF QUININE

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The connection between the acid base balance of the organism and the behavior of certain therapeutic agents has occasionally attracted attention. In the case of quinine as well as of some other alkaloids there is evidence that a direct relationship exists between the amount of the drug excreted in the urine as a result of a standard dose and the acid-base balance as indicated by the pH of the urine. Furthermore, various authors have recommended administration of alkalis along with quinine dosage for certain types of malaria on the ground of a higher rate of absorption, higher blood concentrations of quinine, etc. Among such papers may be mentioned those of Acton and Chopra (1) and Sinton (2) in which are included references to other papers with similar recommendations, based largely on clinical results. More recently Guilosirova-Guryea (3) re-reported that an alkaline dietary regime caused a delay in the onset of quinine excretion after dosage and a corresponding delay in its cessation. This implies that administration of alkali or an alkaline regime merely delays excretion of urinary quinine but does not affects its total quantity. The method of testing reported by the above author was however, essentially qualitative in nature. He concluded that an alkaline condition is to be recommended in malaria therapy on the ground that retention of the quinine within the organism for a longer period would necessarily be beneficial.

As far as the present authors are aware, the first published evidence that the acid base balance of the organism has any influence on the total amount of quinine excreted was that of Haag Larson and Schwartz (4). These authors working with human subjects demonstrated that within a 48-hour period, an acid regime produced an excretion of about twice the percentage of the quinine dosage as did an alkaline regime. Moreover the separate figures for percentage excretion for the second 24-hour period were on the average slightly lower under alkaline than under acid conditions, a result contrary to what one would expect if the effect were merely a deceleration of excretion as indicated by Guilosyova Guryea.

Haag Larson and Schwartz explain their data on the basis of resorption of the alkaloid from the urinary tract the process proceeding to a greater extent with an alkaline urine. Such a mechanism seems to the present authors unlikely and before accepting it one would prefer to eliminate other possibilities. Among these may be listed first that variations in the pH of the intestinal tract might influence the rate or degree of absorption of the quinine salt second that variations in the acid base balance of the whole organism might influence either the

rate of absorption or the degree of metabolic decomposition and hence the extent of urinary excretion of the unchanged alkaloid, third, that variations in the pH of the urine might influence the keeping qualities of the quinine excreted in it

The third possibility, of those listed above, may be disposed of briefly. We have compared the quinine content of various samples of urine over a wide range of pH values over periods of days and weeks. Using the analytical method of Kyker, Webb and Andrews (5) we have failed to detect any change in the apparent quinine content of the urine if protected from light during the period of preservation.

The question as to the possible effect of acid-base balance on the rate of absorption and metabolism of quinine salts was studied by the use of dogs with isolated intestinal loops. These dogs were prepared and used as described in recent papers from this laboratory (6, 7). The great advantage of the use of isolated intestinal loops lies in the fact that, with fecal loss prevented, much more quantitative relations are possible between that part of the dose actually absorbed and that excreted. All doses of quinine salts placed in the loops were based on 20.0 mgm anhydrous quinine sulfate (or 17.36 mgm as the free base) per kgm of body weight. All determinations of quinine, whether on the material washed from the loop or on urine samples, were made by the method of Kyker, Webb and Andrews (5).

The effect of the acid-base balance of the animal on absorptions from isolated loops may be studied in two ways. The acid or base may be administered (within limits) in the loop, along with the quinine, or acidic or basic agents may be administered by mouth to the animal while the quinine salt in water solution or suspension is placed in the loop. In the work here reported both procedures were used. It is obviously impossible to cause very wide variations in the pH of the loop contents without damage. The effect on such absorption speeds of adding equivalent amounts of alkali to either quinine dihydrochloride or sulfate in the loops has already been reported (7). It was observed at that time that although the free base when introduced as such, was absorbed much less rapidly than the dihydrochloride and somewhat less rapidly than the sulfate, much more irregular results were obtained in some cases when the latter salt was placed as such in the loop along with an equivalent amount of alkali. In general, however, it may be said that addition of equivalent base in the loop to either quinine sulfate or dihydrochloride produced the lower rate of absorption previously observed for suspensions of the free base. The effect of amounts of sodium hydroxide larger than those equivalent to the alkaloid was tested in loop dogs with the result that no change in the rate of absorption was observed. The excess sodium hydroxide, over that required to convert the dihydrochloride into the base, consisted of 0.215 milliequivalents in a total volume of 20 ml.

Variations of an acidic nature were tried by addition of 0.5 gm ammonium chloride in solution to the loop after adding the standard dose of quinine dihydrochloride. This mixture should produce an initial pH in the loop contents of about 5.0. The results in all cases showed a definite decrease in rate of absorp-

tion For example on Dog I 30 minute experiments with the dihydrochloride in water solution gave absorption percentages averaging 26% With the ammonium chloride added this figure was reduced to 19.6%. Practically no effect was observed in experiments with quinine sulfate. Similar comparisons in 60-minute experiments showed no significant change in absorption rate It would seem that whatever influence the ammonium chloride might exert was nullified by its more rapid absorption. It is of course obvious that such experimentation is limited by the possibility of damage to the loop by too high concentrations of either acid or alkali but there is certainly no evidence that higher acidity causes any increase in absorption rate and could therefore not account for the higher urinary excretions observed under acid conditions

The effect of oral ingestion of alkali or of acid forming materials was next investigated, both as regards the rate of absorption from the intestinal loops and as regards the proportion of the absorbed dose excreted in 48 hours The four different animals used for these experiments were the same ones referred to in the recent report of Cornatzer and Andrews (7) Quinine dihydrochloride was used in all cases in the standard dosage mentioned above and 60 minute absorption periods were used

Table 1 shows the data obtained from this series both as regards percentage absorption from the loops and percentage of the absorbed drug excreted in the urine in two separate 24 hour periods In most experiments oral administration of sodium bicarbonate or of ammonium chloride were used to obtain alkaline or acid urines. However the nauseating effect of ammonium chloride on the dogs often made its retention difficult and ammonium benzoate was later substituted with somewhat more satisfactory results. Under the best of conditions it was not found possible in any case to produce a urinary pH as low as 6.0 The dose of acid or alkali forming material was administered to the animals for two days previous to the administration of the quinine and was continued during the 2 24-hour periods of urine collection In the case of each animal an average value of the percentage absorption of the same dose of quinine dihydrochloride under normal conditions during 60 minutes is included for purposes of comparison.

Urine collections were made by catheterization and the dogs were kept in metabolism cages permitting complete collection of all urine voided at other times Although collections were continued for two 24-hour periods, it is obvious that only comparatively small amounts of quinine were excreted during the second day Goodman and Gilman (8) state that with rats the excretion of a single dose of quinine is practically complete within 24 hours It seems safe to conclude that 48 hours collection of urine obtains practically all quinine excreted and we find no foundation for the idea that alkali therapy markedly delays quinine excretion

We do however confirm the findings of Haag, Larson and Schwartz as to the lowered percentage excretion resulting from alkali The total percentage excretions listed in Table 1 are, as a rule, about twice as great after administration of acid as after administration of alkali. The more irregular figures obtained with

Dog IV have appeared to be the result of a persistent infection in this particular loop. It is doubtful if the two lower figures recorded for quinine excretion after sodium bicarbonate dosage should be credited.

TABLE 1

Effect of oral dosage of acid and alkali on the absorption of quinine dihydrochloride from intestinal loops of dogs and its urinary excretion. All absorption experiments were of 60 minutes duration. Urine collections were made during two separate 24 hour periods. All doses are in terms of 20.0 mgm anhydrous sulfate per kgm body weight but are expressed in the table in terms of the free base.

DOG NO AND DOSE AS FREE BASE	SALT GIVEN ORALLY	PER CENT QUININE AB- SORBED	URINARY EXCRETION								TOTAL PER CENT EX- CRETED
			1st 24 hours				2nd 24 hours				
			pH	Vol.	Quinine ex- creted	Per cent excreted	pH	Vol.	Quinine ex- creted	Per cent excreted	
I 0.2466 gm	None	35.1		cc	mgm			cc	mgm		
	NH <sub>4</sub> Cl	46.3	7.3	600	3.73	3.26	8.5	1240	2.08	1.82	5.08
	NaHCO <sub>3</sub>	52.0	8.4	560	2.38	1.85	8.6	465	0.93	0.72	2.57
II 0.3882 gm	None	42.3									
	NH <sub>4</sub> Cl	51.5	6.1	2000	9.58	4.79	7.8	1200	1.68	0.84	5.63
		53.2	7.4	910	8.53	4.10	8.4	1058	2.59	1.25	5.35
	NaHCO <sub>3</sub>	43.0	8.8	900	3.13	1.87	8.9	590	1.10	0.65	2.52
49.4		8.8	815	6.03	3.14	8.9	685	1.39	0.72	3.86	
III 0.1789 gm	None	72.2									
	Ammonium benzoate	74.9	6.1	440	6.20	4.60	6.2	370	0.59	0.42	5.02
	NaHCO <sub>3</sub>	69.4	7.9	428	3.53	2.80	8.7	710	1.11	0.89	3.69
IV 0.2362 gm	None	96.1									
	Ammonium benzoate	87.9	6.3	450	27.27	13.10	6.4	400	0.98	0.47	13.57
	NaHCO <sub>3</sub>	86.3	7.7	330	1.68	0.82	7.9	300	0.60	0.29	1.11
		78.2	7.4	410	11.29	6.10	7.4	450	0.83	0.44	6.54
		93.9	7.7	510	2.52	1.20	7.5	460	0.77	0.34	1.54

## CONCLUSIONS

Based on the amount of quinine actually absorbed from the loops, an acid regime produces a percentage excretion of the unchanged alkaloid about twice as great as that produced by an alkaline regime. We thus confirm, by a different

technique the observations of Haag Larson and Schwartz. Consistent differences in absorption do not appear to provide an explanation for this result

The authors wish to acknowledge the assistance of the Samuel S. Fels Fund in providing means for carrying out this work.

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## STUDIES ON SYNTHETIC CURARE-LIKE COMPOUNDS

### III TOXICITY AND CURARIZING ACTION OF SOME NEW QUININE DERIVATIVES<sup>1</sup>

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The use of quinine as a musculo-relaxing agent in myotonia (1) has emphasized the clinical value of this alkaloid in the therapy of chronic neuromuscular spasm. Similarly the use of quinine methochloride to ameliorate the untoward osseous and muscular injuries resulting from the convulsive treatment of schizophrenia (2) has demonstrated the probable usefulness of quinine derivatives in the role of acute paralyzants.

An earlier report (3) concerning the curare-like properties of a group of quinine and other quaternary ammonium compounds revealed that, of the synthetic compounds studied, the quinine derivatives were the most promising. Quinine ethochloride dihydrate was most effective, quinine methochloride dihydrate merited special mention. Because of the increased curarizing action of the ethyl over the methyl derivative it was thought desirable to investigate additional congeners. Seven new quinine derivatives were studied for toxicity and curarizing action. Certain data concerning quinine ethochloride, quinine methochloride, and the erythrina alkaloids, beta-erythroidine hydrochloride and dihydro beta erythroidine hydrobromide<sup>2</sup> were adapted from previous reports (3 and 4) and further observations made for purposes of comparison. Corresponding investigations were also made with curare (Merck Lot No 32773) and a few observations with quinine hydrochloride.

The new synthetic quinine derivatives examined were as follows:

Quinine n-propyl bromide  
Quinine n-propyl chloride  
Quinine isopropyl chloride  
Quinine n-butyl chloride  
Quinine n-amyl bromide  
Quinine isoamyl chloride  
Quinine hexyl bromide

**ACUTE TOXICITY** Acute toxicity following intravenous administration of one per cent aqueous solutions of the twelve substances under consideration was studied by employing 267 rats, 206 rabbits and 145 dogs. From three to fifteen rats, two to nine rabbits, and one to ten dogs were used at each dosage level. The data thus obtained was analyzed according to the method of Reed and Muench (5) for the determination of fifty per cent end points and is presented in table 1.

<sup>1</sup> This research was supported by a grant from Parke, Davis & Company, in whose laboratories the quinine derivatives were synthesized.

<sup>2</sup> Supplied by courtesy of Dr. D. F. Robertson of Merck & Company.

The toxicity of the quinine compounds for rabbits was roughly twice as great as for dogs with the exception of quinine isopropyl chloride which was characteristically irregular in other categories also. The *n* propyl bromide and chloride were of approximately equal toxicity and were the most toxic of these synthetic agents. The hexyl and isoamyl derivatives were the most innocuous. As a point of comparative interest quinine hydrochloride was injected intravenously in doses of 50, 75 and 100 mg./kg. into three dogs and in doses of 25 and 37 mg./kg. into two rabbits. All animals except that dog on 50 mg./kg., showed stimulatory symptoms and died. There were no indications of paralysis or muscular

TABLE 1  
Toxicity following intravenous administration

COMPOUND	RATS				RABBITS				DOGS			
	Doses employed			LD <sub>50</sub>	Doses employed			LD <sub>50</sub>	Doses employed			LD <sub>50</sub>
	Range	Number	Total number animals		Range	Number	Total number animals		Range	Number	Total number animals	
Q = Quinine	mg./kg.			mg./kg.	mg./kg.			mg./kg.	mg./kg.			mg./kg.
Q metho Cl	2.5-10.0	5	25	5.0	5.75-12.5	5	12	7.0	10.0-20.0	4	12	16.3
Q etho Cl	5.0-7.5	4	20	5.2	5.0-8.0	6	17	7.6	5.0-15.0	8	18	12.0
Q <i>n</i> propyl Br	2.5-5.0	6	32	4.2	3.25-5.0	4	11	3.4	4.5-7.5	6	29	5.9
Q <i>n</i> propyl Cl	4.5-7.5	3	11	6.9	2.0-5.0	7	33	2.0	3.0-5.0	4	8	4.3
Q isopropyl Cl	15.0-25.0	3	15	20.8	10.0-20.0	3	9	13.2	5.0-20.0	4	7	0.3
Q <i>n</i> butyl Cl	6.0-10.0	3	15	7.2	5.0-10.0	4	10	5.8	5.0-15.0	6	13	9.5
Q <i>n</i> -amyl Cl	3.0-5.0	3	15	4.5	5.0-12.5	5	14	10.0	10.0-17.5	4	9	15.6
Q isoamyl Br	5.0-7.5	4	20	5.3	5.0-12.5	6	15	10.3	10.0-25.0	5	12	20.0
Q hexyl Br	7.5-10.0	5	25	9.3	7.5-12.5	5	19	10.0	10.0-25.0	6	12	20.8
Beta-erythroidine	35.0-50.0	3	30	39.3	5.0-12.0	8	22	8.6	5.0-10.0	3	8	8.8
Dihydro-beta-erythroidine	0.5-15.0	6	49	8.0	1.0-2.5	7	29	2.1	1.0-2.0	3	9	1.1
Curare					0.75-1.5	5	15	1.3	1.0-2.0	4	8	1.2

\* Calculated by the method of Reed and Muench (5)

weakness. The doses of beta-erythroidine and curare causing death of fifty per cent of animals (LD<sub>50</sub>) were approximately the same in rabbits and dogs. Dihydro beta-erythroidine was twice as toxic for dogs as for rabbits and was in the same range as curare.

The relative toxicity of the quinine congeners in rats varied from that in other animals. The fifty per cent lethal dose ranged from 4.2 to 9.3 mg./kg. with the exception again of quinine isopropyl chloride of which the LD<sub>50</sub> was 20.8 mg./kg. Of the others quinine hexyl bromide was least toxic. Dihydro-beta-erythroidine fell in the same range as the quinine compounds, but, relatively beta-erythroidine was extremely nontoxic in rats. Its LD<sub>50</sub> was 39.3 mg./kg.

**CURARIZING EFFECTS** The curarizing action of the agents studied was ascertained and roughly quantitated in a series of experiments using frogs, rabbits

and dogs. The seat of paralytic action at the neuromuscular junction was defined by application of the classical Claude Bernard experiment (6) with frogs and the Thomas and Franke experiment (7) with dogs.

The Thomas and Franke technique was modified as previously described by Lehman (8). A description of the modified procedure is included here as an



FIG 1 MODIFICATION OF THE THOMAS AND FRANKE TECHNIQUE

The diaphragmatic strip is moistened by drops of warm oxygenated Locke's solution and the pleural cavity utilized as a moisture chamber. The insert at the lower right depicts in detail the technique of removing the diaphragmatic strip.

explanation of the accompanying illustration (fig 1). The presence or absence of central nervous response to a drug was judged by observing the contractions of a portion of diaphragmatic muscle which was protected from the local action of the drug by excision but retained its connection with the central nervous system by means of its intact phrenic nerve. Simultaneously the agent's pe

ripheral action was noted by recording the contractions of the opposite leaf of intact diaphragm with normal blood and nerve supply. One essential modification of the original technique of Thomas and Franke has been made. Instead of placing the excised pie shaped strip of muscle in a beaker of Locke's solution we fixed the dorsal end to the tip of an L rod reaching down into the pleural cavity and the ventral end to a thread activating a system of tambours for recording. Environmental temperature and moisture were maintained for the strip by bathing it

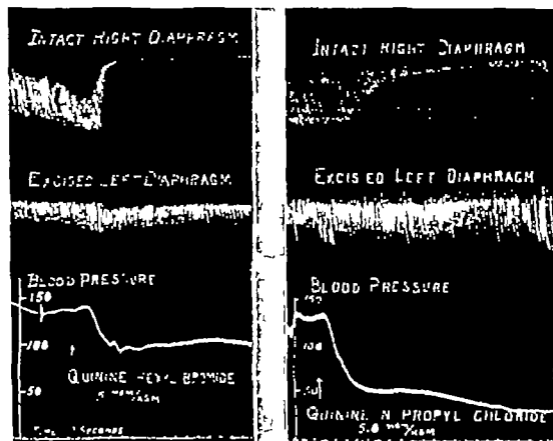


FIG. 2. TYPICAL TRACINGS FROM TWO EXPERIMENTS EMPLOYING THE MODIFIED THOMAS AND FRANKE TECHNIQUE

Quinine hexyl bromide was injected intravenously into the first dog in a dose approximating three-fourths of its  $LD_{50}$ , resulting in immediate cessation of contractions of the right diaphragm and not of the excised strip signifying peripheral but no central effect on the respiratory mechanism.

Quinine n-propyl chloride was injected into the second dog in a dose greater than its  $LD_{50}$  and was only partially effective as a paralyzant.

constantly with warm oxygenated Locke's solution dropped from above. This arrangement allowed the phrenic nerve to lie in a more normal position without tension and utilized the pleural cavity as a moisture chamber. Details of the technique are illustrated in figure 1.

Minimal curarizing doses were determined for frogs and are presented in table 3. In previous studies any substance having a minimal curarizing dose of more than 100 mg./kg. was regarded as relatively ineffective and as not meriting further study. The only member of the present series which exceeded this level

was quinine isopropyl chloride with a minimal paralytic dose of 200 mg/kg. Subsequent experiments demonstrated the validity of the original premise, for this agent showed very inferior curarizing power. Quinine hydrochloride produced no paralysis in frogs in doses as high as one gram per kilogram.

A range of doses giving maximal paralysis of the peripheral respiratory mechanism of the dog without seriously affecting the central respiratory control was determined by the Thomas and Franke method for each compound studied (table 3). Six of the quinine congeners, the erythrina alkaloids and curare showed complete paralysis of the peripheral respiratory mechanism without seriously affecting the central control at doses which were at or below their

TABLE 2  
*Paralytic action at head drop and convulsant levels*

COMPOUND	RABBITS—HEAD DROP ASSAY				DOGS—ANTAGONISM METAZOL CONVULSIONS				
	Doses employed		Total number animals	HDrD <sub>50</sub>	Doses employed		Total number animals	Protection without fatality	
	Range	Number			Range	Number		Dose (PD)	Degree
<i>Q = Quinine</i>	<i>mg/kg</i>			<i>mg/kg</i>	<i>mg/kg</i>			<i>mg/kg</i>	
Q metho Cl	3.0–5.75	7	17	5.0	7.5–15.0	4	14	10.0	3
Q etho Cl	2.5–4.0	6	19	3.3	5.0–12.5	6	40	8.5	4
Q n propyl Br	2.0–3.5	7	14	3.2	3.5–5.0	4	9	3.5	1
Q n propyl Cl	1.0–2.75	6	22	2.4	3.0–4.0	5	6	3.0	1
Q isopropyl Cl	8.0–9.0	3	7	8.6	7.0–8.0	2	2	7.0	0
Q n butyl Cl	3.0–5.0	4	10	4.2	5.0–8.0	6	9	6.5	2
Q n amyl Br	3.5–5.0	5	16	3.0	10.0–14.0	4	9	12.0	4
Q isoamyl Cl	2.5–7.5	7	18	4.3	12.0–15.0	5	11	13.5	4
Q hexyl Br	3.5–5.5	6	31	4.1	10.0–15.0	4	9	11.0	4
Beta-erythroidine	5.0–9.0	6	15	7.6	4.0–5.0	2	4	4.0	1
Dihydro beta-erythroidine	0.75–2.5	8	32	1.5	0.5–5.0	7	11	0.75	1
Curare	0.5–1.0	4	14	0.6	0.75–1.5	5	11	1.0	2

\* Determined by the method of Reed and Muench (5)

† 0 = No protection, 4 = Complete protection

respective LD<sub>50</sub> levels for unanesthetized dogs. Figure 2 illustrates the action of quinine hexyl bromide which was very effective and quinine n-propyl chloride which was only partially effective as a curarizing agent. Quinine isopropyl chloride gave partial paralysis at the dose which killed fifty per cent of animals. Quinine n-propyl chloride and quinine n-butyl chloride gave respectively, almost complete and complete paralysis, but at doses at or above the canine LD<sub>50</sub> for these compounds.

**COMPARATIVE CURARIZING ACTIVITY** The relative curarizing powers of the compounds studied were determined at two levels. First, the level of threshold paralysis was assayed by the rabbit head drop method for curare devised by Holaday (2). The doses giving head drop in fifty per cent of animals (HDrD<sub>50</sub>) were determined in the same manner as were lethal doses (LD<sub>50</sub>) (5). Two

hundred and five observations were made. In many instances the same animal was used for several tests by allowing a two to seven day interval for recuperation between injections. A detailed study of these experiments is presented in table 2. In order to compare the compounds on an equivalent basis the therapeutic index was computed by determining for each substance the ratio of the dose giving head drop in fifty per cent of the animals to the dose killing fifty per cent ( $H\ Dr\ D_{50}/LD_{50}$ ) and expressing the ratios in terms of per cent of the  $LD_{50}$ . These indices appear in Table 3.

TABLE 3

*Relationship of toxic and curarizing doses of quinine compounds erythrina alkaloids and curare*

COMPOUND	PROGS	RATS		RABBITS		DOGS					
	Para-lytic dose	LD <sub>50</sub>	LD <sub>50</sub>	HDrD <sub>50</sub>	HDrD <sub>50</sub> /LD <sub>50</sub>	Thomas & Frauke experiment		LD <sub>50</sub>	Antagon-ists metrazol		PD/LD <sub>50</sub>
						Range doses employed	Degree paralysis peripheral respiratory mechanism		Best protection with recovery		
									Dose (PD)	Degree	
Q = Qui loc	mg./kg	mg./kg	mg./kg	mg./kg	Per Cent	mg./kg		mg./kg	mg./kg	Per Cent	
Q metho Cl	40 0	5 0	7 0	5 0	72	5 0-15 0	4	16 3	10 0	3	63
Q etho Cl	30 0	5 2	7 6	3 3	44	5 0-25 0	4	12 9	8 5	4	66
Q n propyl Br	50 0	4 2	3 4	3 2	93	2 5- 5 0	4	5 9	3 5	1	60
Q n-propyl Cl	70 0	6 9	2 9	2 4	81	5 0-10 0	3	4 3	3 0	1	71
Q isopropyl Cl	200 0	70 6	13 2	8 6	65	10 0	2	0 3	7 0	0	77
Q n butyl Cl	50 0	7 2	5 8	4 2	74	10 0-20 0	4	9 5	6 5	2	63
Q n amyl Br	30 0	4 5	10 0	3 9	39	15 0-20 0	4	15 6	12 0	4	77
Q isoamyl Cl	70 0	5 3	10 3	4 3	41	15 0	4	20 0	13 5	4	68
Q hexyl Br	80 0	9 3	10 0	4 1	41	10 0-15 0	4	20 8	11 0	4	53
Beta-erythroidine	5 0	39 3	8 6	7 6	88	0 5-15 0	3	8 8	4 0	1	46
Dihydro-beta-erythroidine	2 0	8 9	2 1	1 5	73	0 5-20 0	4	1 1	0 76	1	70
Curare	2 5		1 3	0 6	47	1 0- 2 0	4	1 2	1 0	2	73

0 = None 4 = Complete

Four quinine congeners were outstanding by this method of assay, namely quinine ethochloride, quinine isoamyl chloride, quinine n-amyl bromide and quinine hexyl bromide. For all of these the  $H\ Dr\ D_{50}$  ranged from 39 to 44% of their  $LD_{50}$ . This was in the same range of effectiveness as curare for which the  $H\ Dr\ D_{50}$  was 47% of the lethal dose. The erythroidine alkaloids had margins of safety approximating those of the less active quinine derivatives.

The second level for comparing curarizing action was that of optimum paralysis consistent with survival of the animals. This was established by observing the ability of the drugs to protect dogs against the convulsions which followed intravenous administration of a surely convulsive dose (25 mg/kg) of metrazol. The

details of this procedure have been previously described (4) and are omitted here to conserve space. Data defining these experiments appear in table 2. Comparisons were made between protective dose (PD), which was that dose of each compound giving the greatest degree of protection from convulsions without fatality, and the dose killing fifty per cent of dogs (PD/LD<sub>50</sub>), expressed in terms of per cent of the LD<sub>50</sub>. These ratios are presented in table 3 and it is noted that they all fall in the range from 60 to 77 per cent of the LD<sub>50</sub>, with the exceptions of quinine hexyl bromide (53 per cent) and beta erythroidine (46 per cent). What is more important to note is the degree of protection afforded by the protective doses. The same four quinine derivatives mentioned as outstanding by the rabbit head drop method were also most effective in this instance, giving complete protection against metrazol convulsions. By this test, however, curare offered only second degree protection (50 per cent) without death. Quinine isopropyl chloride gave no protection, the erythroidine alkaloids and n-propyl derivatives afforded first degree, and the n-butyl and methyl congeners only second and third degree modification of the convulsive seizures.

**DURATION OF PARALYSIS** Duration of curarizing effect was roughly quantitated by noting the time following intravenous injection of the paralytic agents at which dogs were able to stand unassisted. It is probable that the fall in blood pressure and the general systemic effects on the animals may have been extraneous factors in this crude test. The administration of metrazol made no difference in the recovery times following maximal tolerated and protective doses. Paralysis was apparent for 4-13 minutes following injection of the quinine derivatives, quinine n-propyl chloride, (18 to 45 minutes at 3.0 to 4.0 mg./kg. without metrazol) was the only exception. Dogs recovered after beta erythroidine within 6 to 12 minutes, after dihydro beta-erythroidine in 15 minutes, and after curare in 18 to 33 minutes.

**DISCUSSION** In reviewing the complete picture of the toxicity and curarizing power of the compounds studied as presented in table 3, it is evident that the ethyl, n-amyl, isoamyl and hexyl quinine derivatives were consistently the most effective. The n-propyl and isopropyl compounds were relatively weak curarizing agents and the methyl and n-butyl agents had only moderate action. Quinine hydrochloride was almost devoid of paralytic properties. The erythrina alkaloids were comparable in therapeutic index to the propyl compounds. Although curare had a fairly wide margin of safety as determined by the head drop method of assay in rabbits, it was quite ineffective in safely modifying metrazol convulsions in dogs.

There was no essential difference in the toxicity or curarizing action of chloride and bromide salts of the quinine n-propyl variant. It is reasonable to assume that this might hold true for the other members of the series since the bromide content of a curarizing dose for dogs is only about two per cent of that of a sedative dose of sodium bromide for the same species.

The protective dose against metrazol convulsions, which represented in most instances the maximal tolerated dose under these circumstances, was at or very near the maximal tolerated dose for the curare like drug alone as determined in

the toxicity experiments. This fact coupled with its lack of influence on the duration of action, would suggest that metrazol in the dose used, was neither antidotal to nor did it add to the toxicity of the paralytic agents.

The curarizing action of the quinine derivatives is intense and of short duration. Evidently the lengthening of the aliphatic side chain does not prolong their action, an attribute which might be desirable in treating chronic neuromuscular spasm.

We wish to express our appreciation to Keter Donelson and Paul Gradolph, David Reisman and Thomas Spaulding of the College of Medicine for their technical assistance in this study.

#### SUMMARY AND CONCLUSIONS

1. A group of nine quinine derivatives has been studied for toxicity and curarizing activity and compared in these respects with quinine hydrochloride, two erythrina alkaloids and curare.

2. All compounds produced curare like paralysis in frogs by the Claude Bernard technique except quinine hydrochloride which did not show evidence of paralytic action in this or any subsequent test.

3. All compounds showed peripheral neuromuscular paralysis in dogs as demonstrated by the modified Thomas and Franke technique described in this paper.

4. The descending order of toxic and curarizing efficiency as determined by comparing the fifty per cent head drop and lethal doses for rabbits was as follows: quinine *n*-amyl bromide, *q* isoamyl chloride and *q* hexyl bromide, *q* etho-chloride, *q* isopropyl chloride, *q* methochloride, *q* *n* butyl chloride, *q* *n*-propyl chloride and *q* *n* propyl bromide.

5. Consideration of two factors, first, a comparison of the doses giving maximal protection against metrazol convulsions in dogs without death of the animals with the doses killing fifty per cent of the same species and, second, the degrees of protection achieved by the former doses, showed that the relative efficiency under these conditions was in descending order as follows: quinine hexyl bromide, *q* etho chloride, *q* isoamyl chloride, *q* *n*-amyl bromide, *q* metho chloride, *q* *n* butyl chloride, *q* *n* propyl bromide, *q* *n* propyl chloride and *q* isopropyl chloride.

6. The paralysis produced by the quinine derivatives lasted about one third as long as that caused by curare.

7. The results of the head drop assay in rabbits and the antagonism of metrazol convulsions in dogs correlated well. The head drop assay appears to be a fairly reliable screening test for curare-like action.

8. Curare produced head drop in rabbits with a margin of safety comparable to the most effective quinine derivatives but gave only moderate suppression of metrazol convulsions in a dose at which all animals survived. The erythrina alkaloids were comparable in activity to the weaker quinine derivatives. Quinine hydrochloride showed no paralytic action.

9. Metrazol in convulsive dosage was neither antidotal to nor did it increase the toxicity of the paralytic agents.

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# PHARMACOLOGIC STUDIES OF A NEW VASOCONSTRICTOR 2 NAPHTHYL-(1') METHYL-IMIDAZOLINE HYDROCHLORIDE (PRIVINE OR NAPHTHAZOLINE)<sup>1</sup>

## II VASCULAR AND RESPIRATORY REACTIONS IN THE ANESTHETIZED DOG

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In 1939 Hartmann and Isler (1) correlated the chemical structure of certain imidazoline derivatives with their pharmacologic activities Spurred by this report Miescher and Urech (2) synthesized new members of this family of compounds Preliminary experiments (3) singled out one of these 2 naphthyl (1')-methyl imidazoline hydrochloride as especially worthy of an intensive pharmacologic study This paper reports a second aspect of Privine's pharmacologic behavior, namely the vascular and respiratory responses in the anesthetized dog to the drug administered by various routes

**METHOD** Healthy dogs of varying species sex and weight were used in this study The anesthetics employed were ether urethane and sodium pentobarbital With all animals the vagi were sectioned and the blood pressure continuously recorded in the usual way with a mercury manometer A continuous respiratory recording was secured by inserting a Mendenhall respiratory cannula (4) into the right thoracic cavity and connecting it with the usual recording tambour The drugs employed intravenously in this study with their dose ranges given as always in this paper on a per kilogram basis were Privine (0.0005-1.0 mg) epinephrine (1.25-10.0 micrograms), yohimbine and ethyl yohimbine (1-3 mg) ephedrine (0.5-1.0 mg) Both their order of administration and doses were widely varied Extreme care was exercised in washing each drug completely out of the venous cannula with at least 2-4 cc. of physiologic saline before a subsequent injection Cocaine (10-20 mg) was injected intramuscularly Privine was also applied to various membranes in doses to be stated below

**RESULTS** Our results in experiments with 50 dogs are conveniently summarized under the headings to follow

**Minimal Effective Dose** No attempt was made to determine this exactly, but 7 dogs received an initial injection of one microgram of Privine and their responses varied from a barely discernible purple on the blood pressure tracing to a fleeting maximal rise of 12-15 mm. which never persisted for as long as a minute and which not infrequently was duplicated in extent by the injection of physiologic saline alone Since those dogs which received an initial dose of 5 micro-

<sup>1</sup> This report is part of a project which involves a complete pharmacologic investigation of Privine and related amines and is supported by Ciba Pharmaceutical Products of Summit New Jersey

grams almost without exception gave a definite and persisting rise in blood pressure, it is reasonable to presume that the minimal effective dose lies in the range indicated, namely, from 1 to 5 micrograms

*Privine vs Epinephrine* The relative effectiveness of these two drugs cannot be succinctly summarized by a definitive ratio. To cite but two reasons (1) an effective dose of Privine invariably exerted its effect longer than an equal dose of epinephrine, (2) their comparative effectiveness varied with the dose of each drug chosen for comparison, i.e., in general, the higher the dose of Privine chosen for comparison the less effective did it appear in relation to moderate doses of epinephrine. From our results we have estimated the relative pressor

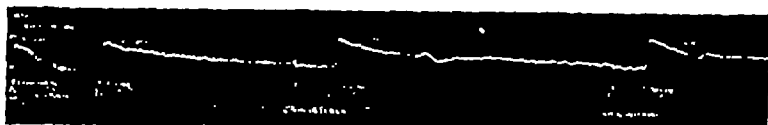


FIG 1 PRIVINE VS EPINEPHRINE DOG, 4 KG, SODIUM PENTOBARBITAL ANESTHESIA  
A, 0.005 mg/kg I V epinephrine, B, C, E 0.025 mg/kg I V privine, H, D kymographic record interrupted 8 and 4 min respectively

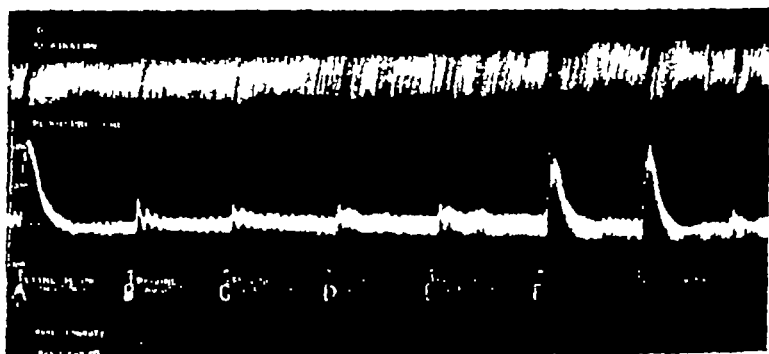


FIG 2 TACHYPHYLAXIS DOG, 7 KG URETHANE ANESTHESIA  
A, F, G 0.005 mg/kg I V epinephrine, B, C, D, E 0.010 mg/kg I V privine

potency of Privine to be from  $\frac{1}{3}$  to  $\frac{1}{2}$  that of epinephrine. The lines of reasoning followed in arriving at that estimate will now be presented, for otherwise the estimate lacks value.

The ratio of the effectiveness of these two drugs is a composite which summarizes the results obtained from three different modes of comparison. (1) the doses of Privine and epinephrine producing equivalent elevations of blood pressure in the same dog have been compared, (2) the absolute rises in blood pressure produced by equal doses of the two drugs in the same dog have been compared, (3) finally, the average rise in the entire series of experiments produced by varying doses of the two drugs, has been compared in terms of mm rise per microgram of drug injected. Figure 1 illustrates the first mode of comparison, figures 6 and 7A illustrate the second.

To consider the first mode of comparison only twice were equivalent rises obtained during the experiments irrespective of the doses of the two drugs employed within the ranges already stated. In both of these experiments it required 25 micrograms of Privine to match the effect produced by 5 micrograms of epinephrine. In each instance the absolute rise obtained was about 50 mm and represented approximately a 30 per cent rise from the basal blood pressure. In other analogous experiments 25 micrograms of Privine gave but 50 to 80 per cent of the rise obtained from 5 micrograms of epinephrine. Thus in some experiments the test dose of Privine was 5 to 10 times the control dose of epinephrine but not even in those experiments with the two exceptions just mentioned did the Privine produce as great a rise as did the epinephrine. Figures 2 and 3 exemplify the relative effects of larger doses of Privine.

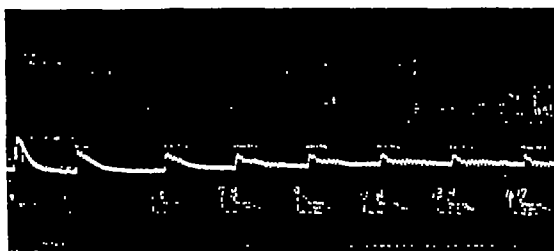


FIG 3 TACHYPHYLAXIS AND RESPIRATORY INHIBITION DOG 9 Kg SODIUM PENTOBARBITAL ANESTHESIA

2 0.002 mg./kg I V epinephrine 3 5 7 0 11 13 16 3.0 cc I V physiological saline  
4 6 8 10 12 14 1 0.020 mg./kg I V privine

To consider the second mode of comparison experiments were considered in which the initial dose of Privine and epinephrine were the same. The response elicited by Privine was then expressed as a percentage of the response produced by epinephrine in the same animal. In 11 experiments the initial 2 injections were 5 micrograms respectively of each drug. The order of injection varied but that was found to be of little significance. In this way we found that 5 micrograms of Privine on the average gave but 38 per cent of the response obtained from 5 micrograms of epinephrine. In this group the increases induced by Privine varied from 20 to 58 mm and those excited by epinephrine from 33 to 110 mm. The effect of the Privine however persisted from 2 to 3 times as long as that of the epinephrine. This prolonged action was the most consistent feature of Privine's behavior. Even in experiments where increasing doses of Privine gave little if any augmentation of the vascular tension over that obtained from smaller doses the duration of the action was yet prolonged in proportion to the dose.

To consider the third mode of comparison for both Privine and epinephrine

the mm rise per microgram of drug injected was calculated for various doses of the two drugs. The curve obtained for each drug by plotting mm rises in blood pressure against doses in micrograms is logarithmic in type. That means that in the range of doses below the toxic level the mm rise per microgram of drug injected decreases as the dose of the drug is increased. To illustrate if 'x' micrograms of either drug produces a blood pressure increase of 'y' mm then 2x micrograms will produce a rise of 'y and z' mm where z is less than 'y'. The mm rise per microgram of Privine injected fell from an average of 6 when the dose was 5 micrograms to somewhat less than 2 when the dose was 25 micrograms. This law of diminishing returns, however, applies as well to epinephrine. Increasing the dose of epinephrine from 2 to 5 micrograms decreased the mm rise per microgram of drug injected from 30 to 15. On this basis of comparison Privine is from 13-20 per cent as active as epinephrine.

*Tachyphylaxis* In 8 animals anesthetized with 1.5 grams of urethane administered per os, no tachyphylaxis was ever demonstrated. In 3 animals anesthetized by ether no evidence of tachyphylaxis was ever demonstrated although for reasons presently to be discussed, but few consecutive doses were given these animals.

In one experiment with a urethanized dog, the animal responded to 50 consecutive doses of 5 micrograms of Privine with steady though brief rises of 10-12 mm up to about the 20th dose when a slight decrease in the response became perceptible, continuing to the 50th dose when the response was but 8 mm. The doses were repeated at about 4 minute intervals. Toward the end of the experiment repeated 50 microgram doses were given the animal. The first response was a 50 mm rise, the second but 10 mm and thereafter no rise could be elicited. At the end of the experiment 3 consecutive 1 mg doses failed to alter the blood pressure and did not kill the animal.

The other 7 dogs anesthetized with urethane were tested for tachyphylaxis with 5, 10, 20 and 25 microgram doses and exhibited none save for one animal which demonstrated it after 5 doses of 25 micrograms. This will be discussed below.

Tachyphylaxis was seen in 8 of 13 dogs anesthetized with sodium pentobarbital but the degree of tachyphylaxis was slight and the final responses in a series of doses in all but two of those animals were significant rises. In those two no responses could be elicited. Figure 3 illustrates the mild tachyphylaxis seen in these dogs. The question might well be raised as to whether this is true tachyphylaxis since the final 5 doses elevated the blood pressure to the same absolute height. The difference in mm rise is due to the fact that each successive dose took off from a slightly higher level because insufficient time was given for a return to the starting level. Nonetheless the first three rises show a progressive, if slight, decrease in the absolute height obtained. In most experiments in which tachyphylaxis was seen after moderate doses (up to 25 micrograms) the degree was not striking.

Figures 1 and 2 are records of experiments in which tachyphylaxis might have been anticipated but did not occur.

In summary, tachyphylaxis was not demonstrated in dogs anesthetized with urethane or ether. A slight degree was inconstantly seen in dogs anesthetized with sodium pentobarbital.

*Effect of Privine on Respiration* From our experiments we have concluded that Privine has no constant or significant effect on respiration. There was a tendency with larger doses (from 20–40 micrograms) toward a temporary respiratory arrest immediately after injection which quickly yielded to a resumption of respiration at the usual rate. This can be seen in figures 3 and 4A. Since this did not always occur and was also seen after some injections of epinephrine (Figures 2 and 3) it was not deemed significant. This change was more often seen in dogs anesthetized with sodium pentobarbital than with urethane. Other respiratory irregularities in the first group were at times clearly precipitated by the injection of a small additional amount of anesthetic as may be seen in figure 4C. In this as in some other experiments (Figures 4A and 4B) such irregularities disappeared during the course of Privine injections. We felt therefore that they reflected the action of the anesthetic rather than that of the Privine. In 3 dogs anesthetized with ether alone each injection, varying from 20 micrograms to 1 mg. was followed after a variable interval of minutes by marked respiratory stimulation and a lightening of the anesthesia. This made the anesthetic level of the animal so variable that tachyphylaxis could not properly be studied.

*Effect of Yohimbine and Ethyl Yohimbine on the Action of Privine* In view of the adrenolytic and sympatholytic actions of these drugs it seemed desirable to test the action of Privine after their administration. We employed both yohimbine and ethyl yohimbine<sup>2</sup> in doses ranging from 1–3 mg. In every experiment controls with epinephrine were used before and after the administration of Privine to determine the yohimbine effect. A blood pressure reversal after the administration of the usual dose of epinephrine was considered evidence for yohimbine's action.

The results with Privine were interesting in that a blood pressure reversal was never realized. The response to an effective control dose of Privine (5–10 micrograms), however, was entirely eliminated in animals which had received effective doses of either of these lytic agents. The response to Privine was not eliminated in toto because doses from 5–10 times the initial control dose always gave an increase in blood pressure of the same order of magnitude as that obtained from the control dose. Figure 5 is typical of these results as obtained in 14 different experiments. The initial dose of yohimbine or its ethyl derivative caused a dramatic fall in blood pressure even after the drug had been given slowly from which the animals did not always recover. In two experiments we gave with the yohimbine, 1 mg. of ephedrine and 50 micrograms of Privine respectively but in neither case was the fall in blood pressure obviated although it was somewhat lessened (fig. 5).

*Effect of Cocaine on Privine's Action.* The results with Privine in 5 cocaineized dogs were equivocal. In 3 animals the response to Privine after cocaine was

<sup>2</sup> Prepared by Dr. W. C. Bywater of Parke Davis and Company.

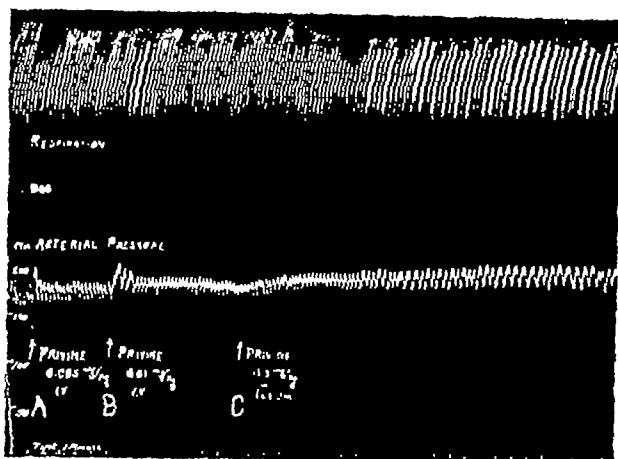


FIG 4A ABSORPTION FROM ILEUM Dog, 10 Kg, SODIUM PENTOBARBITAL ANESTHESIA

A 0.005 mg/kg I V privine, B 0.010 mg/kg I V privine, C 0.300 mg/kg in lumen of ileum

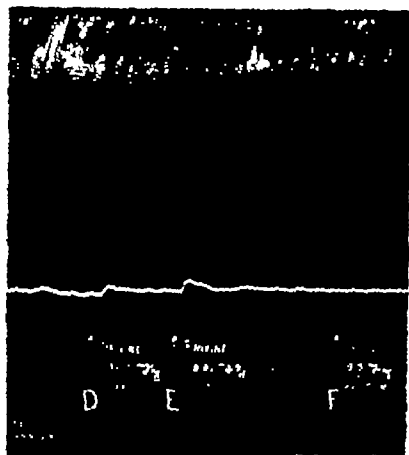


FIG 4B

FIG 4B RECTAL ABSORPTION Dog, 10 Kg, SODIUM PENTOBARBITAL ANESTHESIA  
D 0.005 mg/kg I V privine E 0.010 mg/kg I V privine, F 0.300 mg/kg in lumen of rectum



FIG 4C

FIG 4C RESPIRATORY IRREGULARITIES AFTER PENTOBARBITAL Dog, 10 Kg, SODIUM PENTOBARBITAL ANESTHESIA

G 4.0 mg/kg I V sodium pentobarbital, H 0.3 mg/kg privine in nares

somewhat less than the response to the control dose of Privine and in 2 it was somewhat greater Sensitization by cocaine to epinephrine but not Privine is seen in figures 7A and 7B We tentatively concluded, in view of the slight and

inconsistent changes that cocaine had no constant potentiating or inhibiting action in respect to Privine

**Synergism of Epinephrine and Privine** It would contribute to an interpretation of the mechanism of Privine's action to decide (1) if either drug potentiated the action of the other, (2) if the drugs administered together showed summation in their action or more or less than summation. In none of our experiments was there convincing evidence that epinephrine potentiated the action of Privine. On the other hand two of 50 experiments suggested a potentiation of epinephrine by previously administered Privine. Were this action of Privine a fundamental characteristic it should have been observed more often since all animals received these two drugs at some period of the experiment. This action was inconsistent

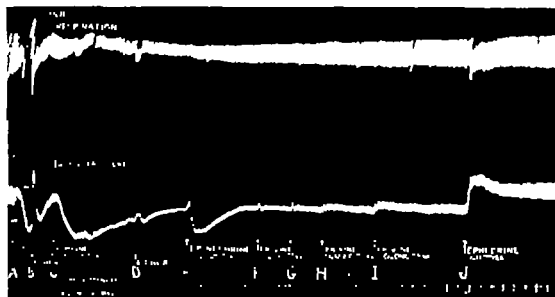


FIG 5 YOHIMBINE INHIBITION OF PRIVINE DOG 7 Kg URETHANE AMESTHESIA

A, C: Ethyl yohimbine hydrochloride to total dose of 1.5 mg/kg I V B D Ether by inhalation E 0.005 mg/kg I V epinephrine; F G 0.010 mg/kg I V privine, H 0.020 mg/kg I V privine I 0.040 mg/kg I V privine J 1.000 mg/kg I V ephedrine

for in the remainder of the experiments the response was no greater than that obtained from the initial control dose of epinephrine (fig 2)

Epinephrine and Privine given together usually gave no more than a summation of responses or even somewhat less than a summation. In two experiments however epinephrine was given with a dose of Privine which immediately prior to their simultaneous administration had failed to elicit any response in the animal. The resultant rise was significantly greater than that obtained from the control dose of epinephrine. It is not possible to say whether in these experiments the Privine potentiated the action of epinephrine or whether the epinephrine resensitized the animal to the action of Privine. In any case the total response in these experiments was no greater than summation if one added the usual epinephrine response to the best previous response to the given dose of Privine. The former hypothesis is more likely because in both experiments a final dose of Privine alone gave no rise in blood pressure. Figure 6 demonstrates

this action. The two responses to Privine shown in this tracing are rather slight. The first two responses of this animal to 0.005 mg. of Privine were respectively 51 and 38 mm. Its initial responses to 0.005 mg. of epinephrine were 65 and 73 mm. It is apparent, therefore, that the responses to the drugs given simul-

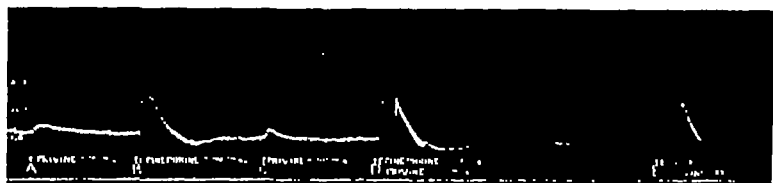


FIG 6 PRIVINE-EPINEPHRINE SUMMATION. DOG, 11 Kg, SODIUM PENTOBARBITAL ANESTHESIA

A, C 0.005 mg./kg. I.V. privine, B 0.005 mg./kg. I.V. epinephrine, D, E 0.005 mg./kg. each of privine and epinephrine given I.V. together

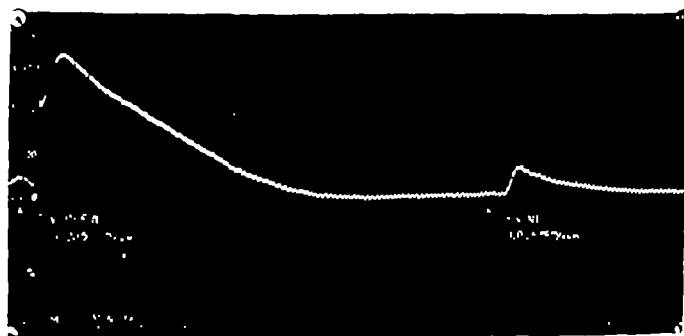
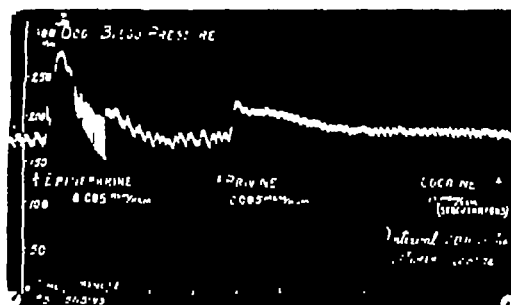


FIG 7A, 7B COCAINE EFFECT ON EPINEPHRINE AND PRIVINE

taneously in that dose, represent no more than summation if one take the sum of the best previous response to each drug

*Mucous Membrane Absorption* In dogs with normal nasal membranes amounts of Privine far in excess of those administered intranasally in clinical practice failed to produce any significant changes in blood pressure. The usual clinical

dose of Privine for intranasal administration would be of the order of 2 drops of 1:1000 in each nostril or roughly a total of 200 micrograms which in the average adult would approximate 3 micrograms on a per kilogram basis. Using this latter basis for comparison the intranasal doses employed with our dogs varied from 3.4 to 208 micrograms. Seven dogs were studied from the standpoint of intranasal absorption. Four of this group showed no detectable blood pressure changes after having received respectively 14, 61, 78 and 208 micrograms. Save for one animal which displayed minor respiratory irregularities after 61 micrograms no changes in their respiratory records were observed. Three other dogs receiving respectively 24, 50 and 159 micrograms showed slow rises in blood pressure of about 20 mm. and the first and last showed respiratory changes. It is thus apparent that Privine can pass the nasal membranes in sufficient amounts to cause moderate systemic changes evidenced by an alteration of the blood pressure level and the nature of the respiration. These changes which required doses many times the usual clinical doses were not constantly seen.

Two attempts to demonstrate a systemic effect from Privine injected into the gastric lumen in doses respectively of 0.55 and 3.9 mg. failed to reveal any kymographic evidence of a systemic effect save for the appearance again of respiratory irregularities in the dog receiving the larger dose. The possibility of rectal absorption of Privine was investigated in four dogs given doses ranging from 1 to 8 mg. No changes in either blood pressure or respiration were seen. Definite evidence of absorption from the small intestine (ileum) was obtained in 2 of 4 animals given amounts varying from 200 to 375 micrograms (fig. 4A). Both of these animals showing blood pressure changes also exhibited respiratory irregularities. The dog which received 200 micrograms showed the highest rise in blood pressure (70 mm. from a level of 150) which we ever witnessed after the administration of Privine. The other rise was insignificant. Evidence of an absorption of the drug when given intraperitoneally was readily secured which was not surprising in view of the known rapid absorption of many drugs so given.

**Discussion.** We estimated the relative pressor effectiveness of Privine to be from  $\frac{1}{4}$  to  $\frac{1}{3}$  that of epinephrine and have previously stated the manner of arriving at that conclusion. Long usage sanctions a comparison on the basis of absolute weight and that tradition has been followed here. It is worth remarking, however, that a strictly rational comparison of the relative effectiveness of two drugs should be made on the basis of mols since in the last analysis the molecule is the basic unit for each drug. The molecular weight of Privine hydrochloride (246.73) is almost exactly  $\frac{1}{3}$  more than the molecular weight of epinephrine (183.20) which means the drugs would be of equal potency if a given weight of Privine hydrochloride produced the result achieved by  $\frac{1}{4}$  of its weight of epinephrine. Two effects of Privine compensate for this relative weakness in comparison with epinephrine: (1) the effect of the Privine usually persisted at least twice as long as that of epinephrine; (2) after the administration of Privine the blood pressure, in due course, dropped back to the starting level but never dropped below it as it usually did briefly toward the end of epinephrine's effect.

It was early demonstrated by Kretschmer (5) that a constant infusion of epinephrine would maintain a constant level of increased blood pressure once an equilibrium had been so established that the rate of infusion equalled the rate of destruction. He and Baylac (6) showed that repeated doses of epinephrine would give the same blood pressure rise. Kreitmair (7) showed for ephedrine and Alles (8) as well as Tainter (9) for benzedrine that repeated doses not only would not give the initial blood pressure rise but a decreasing response to the point where subsequent doses would give a fall. This absence of tachyphylaxis in respect to epinephrine was a characteristic seemingly not shared by other so-called sympathomimetic drugs. It is interesting that in our studies dogs anesthetized with urethane and ether did not exhibit tachyphylaxis whereas, it was occasionally seen in dogs anesthetized with sodium pentobarbital. There was one exception among the urethanized animals which, although we do not feel it represented a true tachyphylaxis, is nonetheless mentioned because of the light it may shed upon the mechanism of tachyphylaxis in the dogs anesthetized with sodium pentobarbital. This animal responded to 5 consecutive doses of 25 micrograms of Privine with blood pressure rises of about 53 mm but the sixth response to the same dose was only 40 mm and the seventh but 28. This animal, however, had shown some respiratory irregularities even before the injection of drugs and by the end of the experiment exhibited Cheyne-Stokes respiration. The seeming tachyphylaxis in this dog may perhaps be better explained on the assumption that persisting hypoxia had diminished the animal's capacity to respond to any drug. This may also, as suggested by Oppenheimer and Hayes (10) account for the mild tachyphylaxis seen in 8 of the 13 dogs anesthetized with sodium pentobarbital. The other 5 did not show it. There are two objections to this explanation as applied to these latter experiments although for the urethanized animal we feel it applicable. (1) the final control dose of epinephrine usually gave the same response as did the initial dose of epinephrine, which would hardly have been the case had developing hypoxia progressively injured the animal, (2) in 2 of the animals a final dose of Privine after a delay of about 25 minutes gave the same response as in the beginning.

It is possible that Privine is synergistic with sodium pentobarbital in causing respiratory depression, this postulation, however, is difficult to accept since Privine never caused respiratory depression in animals anesthetized with urethane or ether. On the contrary, in the 3 animals anesthetized with ether, the injections of Privine were followed after variable intervals of minutes by definite respiratory stimulation. It was not possible to conclude from experiments with only 3 animals whether the Privine was a direct respiratory stimulant or whether it antagonized the action of the ether and so led to a lightening of the stage of the anesthesia with resultant secondary stimulation. We are inclined to the latter view since the corneal reflex was found present at the height of that period of respiratory stimulation and was not present in each instance before it. In about one half of these periods of stimulation the rate at which the ether was being administered had to be increased to avoid convulsions which occurred twice in

the 3 experiments. This manifestation of analepsis may bespeak a central excitant action of Privine or it may even be evidence of an hypoxic response indirectly due to the Privine. Our experiments thus far do not offer a validation of either of these hypotheses. As for the respiratory inhibition seen in dogs anesthetized with barbiturate, just referred to above, we are inclined to exonerate Privine of blame for two reasons which we feel are valid (1) some of the animals anesthetized with sodium pentobarbital became 'light' and small additional amounts of anesthetic were injected on several occasions this injection immediately precipitated the typical respiratory irregularities under discussion and illustrated in figure 4C (2) In the course of some experiments the respiratory irregularities set in and then spontaneously regressed to the point where a normal respiration again obtained, despite repeated injections of Privine throughout the interval (Figures 4A and 4B) A final bit of evidence for this contention that Privine does not inhibit respiration *per se* in moderate doses was the effect of 1 mg. doses of Privine. These either caused very minor respiratory changes or none at all. They never precipitated the 'periodic' breathing seen after sodium pentobarbital.

The failure of Privine ever to produce blood pressure reversal in dogs given effective doses of yohimbine or ethyl yohimbine indicates an essential difference in its mechanism of action from that of epinephrine. In having its action inhibited but not entirely eliminated by the action of these lytic agents Privine resembles the action of ephedrine whose action is partially inhibited after yohimbine as Chase and others (11) have shown and as we confirmed in our own experiments.

Froehlich and Loewi (12) first showed cocaine sensitized an animal's response to epinephrine. Other sympathomimetic drugs have been studied by Hamet, Daly and others (13) from the standpoint of their response after cocaine and some, such as tyramine (in ergotoxinized animals) and arterenol are potentiated by cocaine whereas others such as ephedrine are not. The failure of cocaine unequivocally to sensitize the animal's response to Privine is further evidence for a different mechanism of action from that of epinephrine.

We have been unable to explain why large doses of Privine administered gastrically or rectally failed to yield any kymographic evidence of absorption whereas much smaller doses in the ileum nostrils or peritoneal cavity did. The postulation of an enzymatic destruction of Privine in the stomach and rectum would explain the inactivity of Privine so administered but we have not yet tested this postulation experimentally.

Not infrequently in experiments in which Privine was applied to mucous membranes to determine its absorption therefrom respiratory irregularities were observed after varying intervals with or without attending increases in the blood pressure. This was observed only in animals anesthetized with sodium pentobarbital and in general comprised variations in the extent of the respiratory excursion with concomitant variations in the intervals between respirations. We have already stated why we did not feel this was a manifestation of Privine's

activity It appeared only with doses manyfold those which would ordinarily be applied topically in the clinic. Albert, we do not feel we have excluded a possible adverse synergistic action of Privine and sodium pentobarbital. For the present, therefore, the concomitant use of relatively large amounts of those two drugs might well be attended with caution.

The level of the blood pressure is a reflexion of the algebraic sum of many variables: peripheral resistance, cardiac function, the activities of the vasomotor center, carotid bodies, aortic receptors and still others, some known and some doubtless yet unknown. Ideally we should determine the effect of a given drug upon each of these variables in isolation but in view of the impracticality of this procedure we can only emphasize that the measure of pressor activity we have chosen is a simplified summary of extremely complex phenomena.

Privine has already been shown by Meier and Muller (3) and by Babel (14) to have definite sympathomimetic properties in addition to its general pressor action. It constricts the vessels of mucous membranes when topically applied. Relatively high concentrations cause mydriasis of the somewhat limited and brief type caused by sympathetic stimulants. It was shown to inhibit the movements of the intestines, even those initiated by acetylcholine, it stimulated contraction of the virgin guinea pig uterus. In its action on the coronaries, however, it was unlike epinephrine for it diminished the coronary flow of the isolated rabbit heart, a finding with which two experiments of our own were in agreement.

Our own experiments have not greatly increased the evidence for sympathomimetic action but on the contrary have, in several instances, shown definite divergence from the behavior expected of a sympathomimetic drug. That is not at all surprising since other so-called sympathomimetic drugs have shown such variation. Privine will not produce as great rises in blood pressure as will epinephrine and that bespeaks a different mechanism of action since large doses of Privine (up to 1 mg) at times caused minimal changes in the blood pressure and respiration. Privine is somewhat inhibited but not reversed by yohimbine, and rarely, if ever, potentiated by cocaine. In these two actions it differs sharply from the behavior of epinephrine. It rarely exhibits tachyphylaxis and then usually to but a small degree, so in this respect it more nearly simulates the action of epinephrine than other adrenergic drugs such as ephedrine, which regularly exhibits tachyphylaxis.

To explain the facts presented we have postulated that Privine acts not only on sympathetic endings but on smooth muscle directly. This would explain the ability of an augmented dose to act myotropically beyond endings paralyzed by yohimbine. It would also explain its failure to relax bronchioles in an isolated guinea pig lung (15). The tendency, however, for repeated large doses to exhaust the animal's capacity to respond without, on the other hand, causing very significant changes in the kymographic respiratory or blood pressure recordings, suggests its action rather specifically on a non-vital, exhaustible enzymatic system. The studies herein reported do not warrant a more definitive statement of the mechanism of Privine's action. We hope to elucidate this problem by different approaches thereto.

## SUMMARY

1 Privine elevates the blood pressure in the anesthetized dog in doses of the same order of magnitude as required for epinephrine but to a lesser extent.

2 Privine is partially inhibited but not reversed by yohimbine and ethyl yohimbine, it is not potentiated by cocaine, it infrequently exhibits tachyphylaxis and then only to a slight degree.

3 Privine administered gastrically or rectally in large doses reveals no systemic evidences of absorption. Relatively large doses administered nasally, intra peritoneally, or within the ileum, exhibit minimal systemic changes.

4 Privine may be synergistic with sodium pentobarbital in its depressive action on respiration but no evidence of such depression in dogs anesthetized with ether or urethane could be elicited.

5 The margin of safety for Privine is high since 5 micrograms is an effective dose and 1 mg. failed to kill the animal.

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# ANESTHESIA

## XVI THE DETERMINATION OF ISOPROPENYL VINYL OXIDE IN THE BLOOD OF ANESTHETIZED ANIMALS<sup>1</sup>

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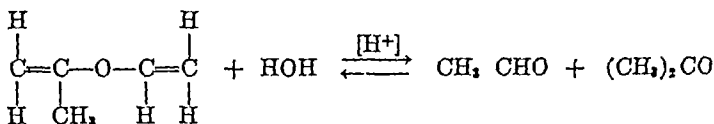
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In a former communication (1) the authors reported studies on the anesthetic properties of isopropenyl vinyl ether (propethylene). It was shown to possess potent anesthetic properties in many species of laboratory animals and also to exhibit a high anesthetic index. Davis and Krantz (2) showed propethylene to be a satisfactory anesthetic in man. The anesthetic syndrome was similar to that produced by ethyl ether. However, propethylene is three to four times more potent and the period of recovery from its anesthesia is especially short and uneventful. Evans, Carr and Krantz (3) showed that repeated and long anesthesias with propethylene in the mouse, rat and dog produced no significant kidney or liver damage. Carr, Kibler and Krantz (4) developed a method to determine quantitatively propethylene in aqueous solution.

These studies are concerned with the application of this method to the blood of anesthetized animals. Further the blood concentrations and the degrees to which propethylene hydrolyzed in the blood of the dog and the monkey are determined.

**THEORETICAL CONSIDERATIONS** Propethylene is stable in neutral and alkaline solutions. In acid solution, however, propethylene is rapidly and completely hydrolyzed according to the following equation (4)



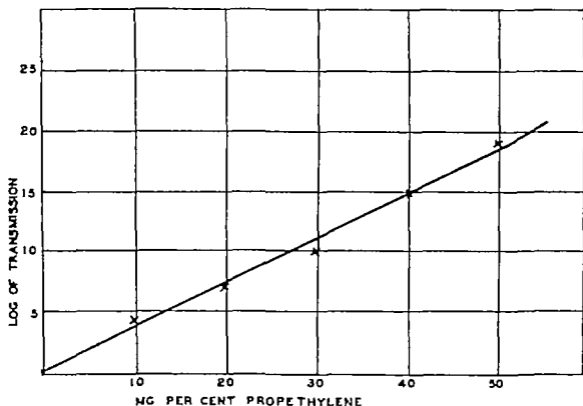
This irreversible decomposition of propethylene into acetic aldehyde and acetone formed the basis for its quantitative determination in blood. The newly formed acetone can be determined in blood filtrates by the method of Behre and Benedict (5). This method depends upon the reaction of acetone with salicylic aldehyde to form dihydroxydibenzene in alkaline solution. The newly formed compound possesses an orange color which permits colorimetric evaluation.

The problem was somewhat more difficult when under anesthesia. Both acetone resulting from the hydrolysis of propethylene *in vivo* and unhydrolyzed anesthetic were to be determined. This was accomplished by determining the total acetone in a Folin-Wu blood filtrate. This value consisted of acetone

<sup>1</sup> The expense of this investigation was defrayed in part by a grant from the Ohio Chemical and Manufacturing Co. of Cleveland, Ohio.

hydrolyzed *in vivo* and all of the acetone obtained from the complete *in vitro* acid hydrolysis of propethylene. Another determination of acetone existing in the blood was made using neutral zinc sulfate and sodium hydroxide as precipitants. This filtrate contained undecomposed propethylene, which does not react with the Behre Benedict reagent, thus enabling one to determine acetone formed in the animal.

**METHOD OF PROCEDURE.** Acetone from blood filtrates was completely recoverable. A standard acetone solution was prepared in water and after reaction with the reagent a standard curve was prepared plotting acetone in concentrations in milligrams per cent against the logarithm of light transmission as meas-



GRAPH 1

ured by a Fisher Electrophotometer. The relationship was linear. From Folin Wu blood filtrates the stoichiometric quantities of acetone resulting from the hydrolysis of propethylene were not found to be present by the method. The method, however, was capable of reproducibility. Accordingly a curve was constructed by adding known quantities of propethylene to shed oxalated blood and determining the total acetone in Folin Wu filtrate by the foregoing procedure. Chart 1 shows this graph, each point representing an average of 5 or 6 determinations.

Blood containing known quantities of propethylene were analyzed and from this curve values were obtained  $\pm 1$  mg. per cent of the actual quantity present.

**ANESTHETIC CONCENTRATIONS OF PROPETHYLENE IN DOG'S BLOOD.** Dogs were anesthetized with propethylene using the closed circuit method with oxygen.

Control acetone determinations were made on the blood of each animal. Blood samples were taken subsequently at 30 and 60 minutes during anesthesia and 15 minutes after the anesthetic cone was removed. These values are shown in table 1.

A series of 3 Rhesus macacus monkeys was anesthetized for a period of one hour. At that time the average quantity of propethylenes present in the blood was 27 mg per cent.

**DISCUSSION** An examination of the data in table 1 shows that upon the inhalation of propethylenes for considerable periods of time the acetone content of the blood is increased approximately 3 fold. Two dogs were anesthetized for 3 hours and the acetone content of their blood was not higher than at the end of the one hour period. A dog was anesthetized with ether for one hour and a

TABLE 1  
*Propethylenes in dog's blood*

DOG NO	SEX	WT	CC./KG PER HR	ACE TONE NORMAL	ACETONE 30 MIN ANESTHESIA	PROPETHYLENE 30 MIN ANESTHESIA	ACETONE 60 MIN ANESTHESIA	PROPETHYLENE 60 MIN ANESTHESIA	ACETONE 15 MIN AFTER ANESTHESIA	PROPETHYLENE 15 MIN AFTER ANESTHESIA
		kg		mg %	mg %	mg %	mg %	mg %	mg %	mg %
1	F	5.75	1.3	0.6	0.9	17	1.5	31	0.4	14
2	F	5.20	1.6	0.5	1.1	30	1.8	45	2.1	21
3	F	6.20	1.7	0.0	0.3	22	1.2	35	1.2	20
4	M	5.80	1.4	0.0	1.2	32	0.8	25	1.2	14
5	F	4.30	1.8	0.3	1.2	25	1.1	33	2.4	22
6	M	5.00	2.1	1.1	1.2	42	2.4	48	2.7	30
7	F	5.20	1.7	0.6	1.2	38	lost	lost	lost	lost
8	F	4.90	1.8	1.2	lost	lost	2.1	47	2.4	29
9	F	4.10	2.4	0.3	0.9	30	1.2	34	lost	14
10	F	4.20	2.3	0.6	3.0	42	3.3	45	2.1	lost
Mean			1.8	0.52	1.2	31	1.7	38	1.8	21

30 minute and a 60 minute blood acetone did not differ significantly from the preanesthetic determination. Presumably the acetone found under propethylenes anesthesia results from an incipient hydrolysis of the ether in blood. Apparently equilibrium is rapidly established between propethylenes and the newly formed acetone and acetic aldehyde. Approximately 2.5 per cent of propethylenes was found hydrolyzed in the dog's blood.

#### SUMMARY

1. A method has been devised to determine propethylenes in blood.
2. The anesthetic concentrations of propethylenes in dog's blood lie between 30 and 40 mg per cent. It is somewhat less in the monkey.
3. Propethylenes for the most part is stable in the blood of the dog and monkey. Approximately 2.5 per cent of the blood concentration undergoes hydrolysis within a period of 3 hours.

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# ON A NEW AEROBIC METABOLITE WHOSE PRODUCTION BY BRAIN IS INHIBITED BY APOMORPHINE, EMETINE, ERGOTAMINE, EPINEPHRINE, AND MENADIONE

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It was reported briefly (1) that brain brei (rat, rabbit, guinea pig) produces a substance arbitrarily called *B* which condenses in acid solution with aromatic amines, including *p* aminobenzoic acid and the sulfonamides, to produce a pigment. We now wish to present data concerning the measurement and chemical nature of *B*, and the characteristics of the system which produces it. The production of *B* is evidently metabolic, since it is sensitive to pH, oxygen tension, and the presence of drugs, and does not occur in heated tissue. The evidence at hand suggests *B* to be an aromatic aldehyde or aminopyrimidine derivative, or both.

The production of *B* is markedly inhibited by apomorphine, menadione, emetine, ergotoxin and adrenaline. Pharmacologically, this is a rather miscellaneous group of drugs, and their common chemical action in the present instance, when finally explained, may provide clues as to their actions elsewhere.

**METHODS** *Production of B* Unless otherwise specified, the following standardized conditions were employed. Rats were stunned and decapitated. The brains, freed of the meninges, plus somewhat less than the required amount of medium (see below) were ground in the Waring blender for 2 minutes, and then diluted so that 1 ml. of suspension contained 100 mgm. of brain. Any isotonic medium can be employed. The following, hereafter called saline phosphate, of pH 6, was used: 200 ml. of 0.9 per cent NaCl plus 75 ml. of 2.1 per cent  $\text{KH}_2\text{PO}_4$  plus 25 ml. of 2.1 per cent  $\text{Na}_2\text{HPO}_4$ . About 10 ml. of brain suspension were shaken in a stoppered 125 ml. Erlenmeyer flask at 37.5°C. in oxygen for 80 minutes. Further incubation would have increased the yield of *B* by not more than 15 per cent. The concentration of brain in the suspension (100 mgm. per ml.) gave the optimum production of *B*. The production of *B* per gram of brain increased as the concentration of brain in the suspension decreased. Thus 250 mgm. of brain per ml. was about 50 per cent less efficient than concentrations in the range 50 to 100 mgm. per ml.

*Preparation of Extract for Testing* The standard procedure was to add to the brain suspension one third its volume of 20 per cent trichloroacetic acid, and centrifuge after 2 minutes. The extract thus obtained was slightly opalescent. Stored at 5°C., it lost less than half its activity in 10 days. Whenever extract was diluted for purposes of testing (see below), 5 per cent trichloroacetic acid was employed, and not water.

*Colorimetry* The quantitative determination of *B* was accomplished by two tests, the *arylamine* and the *thiobarbituric acid*, using the Evelyn photoelectric colorimeter. This machine determines the *photometric density* which is equal to  $\log_{10}$  (100/percentage transmission). Since the Beer Lambert law was obeyed under certain conditions, the photometric density was used as a measure of the amount of *B*, a density of, for example, 1, representing 1  $\mu$  unit or  $t$  unit, depending upon which test was employed. Results were also

<sup>1</sup> We wish to thank the Rockefeller Foundation for its generous support of this work.

calculated as concentrations. Example 10 ml of extract representing 1 gram of brain contained 20  $\alpha$ -units hence there were 2  $\alpha$  units per ml or 20  $\alpha$  units per gram of brain

**Arylamine Test** Aromatic amines condense with *B* in acid solution producing absorption peaks in the far blue. A photoelectric colorimeter is required for the measurement of these colors which lie mostly or entirely outside the visible range. The most convenient amine is *p*-aminoacetophenone though *p*-aminobenzoic acid and the sulfonamides are more or less as good.

The test was carried out as follows. The extract was made up to 1.5 ml with 5 per cent trichloroacetic acid in a colorimeter tube. 1 ml of 1 per cent *p*-aminoacetophenone (dissolved in 25 per cent ethanol) was added (or 4 ml of 0.5 per cent *p*-aminobenzoic acid) and

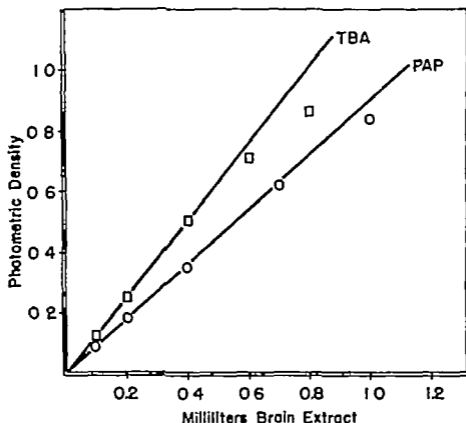


FIG 1 PHOTOMETRIC DENSITY AS A FUNCTION OF QUANTITY OF EXTRACT TESTED  
TBA refers to the thiobarbituric acid test PAP refers to the arylamine test employing *p*-aminoacetophenone

the tube allowed to stand at room temperature for 30 minutes. The tube was then made up to 10 ml with water and read within an hour against a reagent blank using filter 400. The reagent blank contained trichloroacetic acid reagent and water but no extract. An extract blank was not necessary. Under the standardized conditions brain produced about 15  $\alpha$ -units per gram.

The relationship between amount of extract and photometric density is shown in figure 1 for *p*-aminoacetophenone. The absorption spectrum of the pigments developed from *p*-aminoacetophenone and *p*-aminobenzoic acid are shown in figure 2. The former has its maximum at 410 the latter at 890 millimicrons. They were determined with the Coleman photoelectric spectrophotometer using the 30 millimicron slit.

The arylamine test was carried out at various reactions between pH 1 and 10. Maximum color developed between pH 1.5 and 2.5 which is obtained in the standard procedure by the use of the specified amount of trichloroacetic acid. The intensity of color falls off very rapidly above pH 3 so that at pH 7 it is less than 5 per cent of the maximum.

**Thiobarbituric Acid Test** Thiobarbituric acid was found to be a reagent specific for the determination of sulfadiazine among the sulfonamides (2), and essentially the same procedure is used for substance B extract is made up to 1 ml with 5 per cent trichloroacetic acid, 4 ml of thiobarbituric acid reagent (see below) are added, and the solution heated in the boiling water bath for 5 minutes. On cooling, 5 ml of water are added, the solution transferred to a colorimeter tube, and read against a reagent blank, using filter 520. The reagent blank is prepared like the unknown, but contains 1 ml of 5 per cent trichloroacetic acid instead of diluted extract. An extract blank is not necessary. The relationship between photometric density and the amount of extract tested is shown in figure 1.

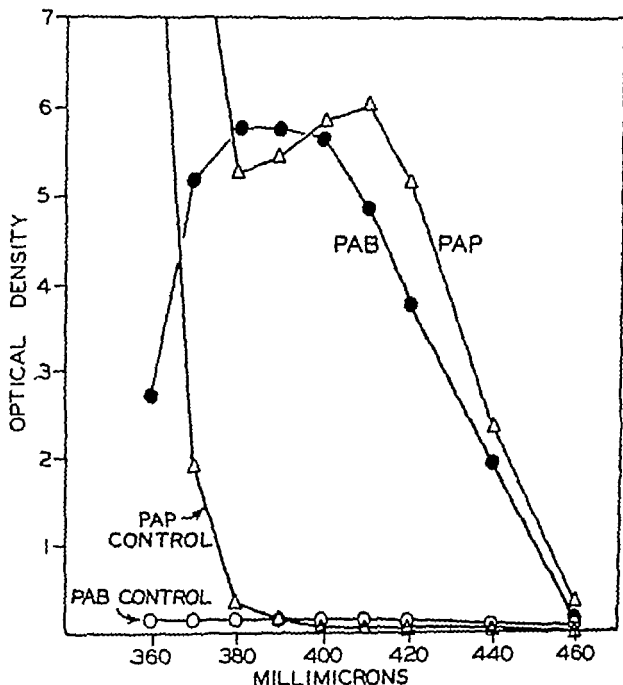


FIG 2 ABSORPTION SPECTRUM OF THE PIGMENT IN THE ARYLAMINE TEST  
The test was carried out using p-aminoacetophenone (PAP curve) and p-aminobenzoic acid (PAB curve)

Thiobarbituric acid reagent was prepared from a 1 per cent thiobarbituric acid solution and a 0.5 M citrate buffer. The former is obtained by mixing 2 grams of Eastman Kodak thiobarbituric acid, 193 ml of water, and 6.6 ml of 2 N NaOH. After heating for several minutes, a more or less dirty yellow solution is obtained, depending upon the batch of thiobarbituric acid purchased. The solution is acidified by adding 0.7 ml of 4 N HCl, and decolorized by shaking for several minutes with 50 mgm of charcoal (e.g., Darco G-60, Merck's U.S.P.). After repeating the charcoal extraction 3 or 4 times, the solution is crystal clear, and either colorless or a faint yellow green. The citrate buffer contains 59 grams of reagent  $\text{Na}_2\text{C}_2\text{H}_3\text{O}_7 \cdot 2\text{H}_2\text{O}$  plus 50 ml of concentrated reagent HCl, is made up to 400 ml with water, and is filtered. The completed reagent is made by mixing 2 parts of thiobarbituric acid with 1 part of citrate buffer, and, if necessary, adjusting the pH to 2.6.

When for purposes of comparison it was necessary to test sulfadiazine, a stock containing

0.5-2 mgm per 100 ml was prepared in 0.2  $\text{N}$   $\text{HCl}$  1 ml of stock plus 4 ml of reagent were heated for 45 minutes. In the case of 2-aminopyrimidine a similar stock was employed, but the heating was continued for 135 minutes.

The absorption spectrum of the pigment developed in the thiobarbituric acid test was determined with the Beckman photoelectric spectrophotometer. The peak of absorption was at 532 millimicrons. Taking the extinction at this wavelength as equal to 100 the relative extinction for other wavelengths was calculated. The following are representative data: 500 millimicrons 6.550 27.540 76.5 532 100 525 84 520 65 500 30 400 5. The curve obtained by plotting these values for substance *B* superimposes on that for either sulfadiazine or 2-aminopyrimidine. The significance of this will be discussed below. The specificity of the thiobarbituric acid test has been studied. Briefly the test will also produce pigments from certain aromatic aldehydes (3) but these absorb in the blue and are readily distinguished in other ways (2) from those of 2-aminopyrimidine and sulfadiazine. Methylation or oxidation of the pyrimidine ring blocks the test.

If we assume that *B* and sulfadiazine have equal extinction coefficients the amount of *B* in brain can be approximated. Brain produces a photometric density of 20 per gram. Sulfadiazine produces a density of 37.5 per mgm. Hence brain contains about 0.5 mgm of *B* per gram.

**Parallelism Between Tests.** Evidence was obtained that both tests measure the same molecule though reacting at different loci. This evidence falls under two headings.

In the first place the tests interfere with one another and are able to reverse each other. This was demonstrated by suitably adjusting the concentration of the arylamine and thiobarbituric acid reagents and by making use of the fact that the pigments developed from them have widely separated absorption bands. Thus in a given preparation the absorption through filter 400 represents the arylamine moiety whereas that through filter 530 represents the thiobarbituric acid. For example by testing separate aliquots with either 1 mgm of *p*-aminoacetophenone or 2 mgm of thiobarbituric acid a diluted extract was found to assay at 0.22 *a*-units or 0.33 *t*-units per ml. A third aliquot was treated first with the arylamine for 30 minutes then with the thiobarbituric acid and finally was made up to 10 ml and read in the colorimeter through the 400 and 530 filters. It assayed at 0.06 *a*-units and 0.22 *t*-units per ml. Hence the thiobarbituric acid test reversed much of the arylamine and neither was able to develop full color. This indicates that both tests deal with the same molecule which is consistent with the fact detailed later that drugs inhibiting the production of *B* always do so by both tests.

On the other hand the ratio of (*t*-units)/(*a*-units) varies somewhat under various conditions. For example in 6 consecutive extracts prepared and tested by the standardized procedure the ratio varied from 1.23 to 1.47. When retested after aging at 5°C for 10 days the ratio had increased significantly in every case the range being from 1.5 to 2.3. Evidently each test measures a different group the arylamine reacting with the less stable

**Isolation.** The pigments developed in the arylamine or thiobarbituric acid tests can be extracted into isoamyl alcohol but not ethylacetate, ether, chloroform or toluene. Dilute alkali will remove them from the isoamyl alcohol. A concentrated thiobarbituric acid derivative can be prepared by adding about 4 mgm of thiobarbituric acid per ml. directly to the trichloroacetic acid extract and boiling for about 10 minutes. The solution is then strongly alkalinized whereupon the pigment becomes deep purple and separates completely. The precipitate can be redissolved in dilute acid. The removal of the pigment from the alkaline solution is due to its absorption on a precipitate. The pigment can be kept in alkaline solution by the following procedure: trichloroacetic acid extract is strongly alkalinized and the colorless precipitate removed. The pH is then brought to 1.5-2.5 the color developed by the addition of thiobarbituric acid and the pH rendered alkaline.

**EXPERIMENTAL RESULTS.** The influence of a number of variables upon the accumulation of *B* was studied utilizing the standard conditions described under Methods. It is of some general interest that *B* is found in appreciable quantities

only following the incubation of the tissue. Freshly taken brain, for example, never contained more than 20 per cent of the amount produced after incubation, and usually contained less than 10 per cent. Evidently *B* accumulates only under the abnormal conditions of incubation. Two mechanisms could be responsible: either the abnormal conditions make possible the formation of *B*, or they inactivate the system which normally removes *B* as rapidly as it is formed.

The effect of pH was checked in a number of experiments. The maximum production of *B* was obtained with an initial pH in the range of 5.6 to 6. Taking this equal to 100, the relative production at other reactions was: pH 4.5, 60; pH 6.5, 75; pH 7.2, 65; pH 8.0, 30. During the course of incubation, the initial pH rose or fell several tenths of a unit in the direction of pH 6 to 6.5.

The effect of oxygen tension is shown in table 1. Two different concentrations of brain were employed: 50 and 100 mgm. per ml. The oxygen consumption was measured in the Warburg apparatus at 37.5°. The table shows that the respira-

TABLE 1  
*Effect of oxygen tension*

The oxygen consumption of two concentrations of brain brei was measured in the Warburg apparatus at 37.5°, in air and in oxygen. The suspending medium was saline phosphate, pH 6. At the end of 80 minutes, the suspensions were washed out of the vessels, and the production of *B* determined by both tests.

CONCENTRATION OF BRAIN (FRESH WEIGHT)	GAS	O <sub>2</sub> CONSUMPTION	UNITS OF <i>B</i> PER GRAM OF BRAIN	
			Arylamine	Thiobarbiturate
mgm./ml		ml./gram		
100	Air	0.97	8.5	10
	O <sub>2</sub>	0.93	12.8	16
50	Air	0.79	8.4	11.2
	O <sub>2</sub>	0.74	12.3	16.7

tion was practically unchanged when oxygen was substituted for air, establishing that diffusion was not a limiting factor in the rate of respiration. The table also shows that the rate of respiration (per gram of tissue) fell about 20 per cent upon diluting the suspension from 100 to 50 mgm. of brain per ml. The behavior of the respiration with respect to dilution and oxygen tension contrasted with that of the system responsible for the accumulation of *B*. The use of oxygen increased *B* by 50 per cent, while dilution was without effect. It is curious that oxygen is necessary for the production of *B*, although, as just noted, no oxygen consumption appears to be involved. Perhaps the production of *B* does not involve the oxidation of a precursor, but only the oxidative activation of an enzyme. In any event, this experiment establishes that oxygen tension can influence the metabolism of brain independently of the oxygen consumption.

The production of *B* was not peculiar to brain, although the latter was more active and uniform in this property than the other rat tissues tested. This is illustrated by the following data for several groups of animals, expressed in *t*-

units per gram brain 20.6, 22.0 19.8 liver 21.4 13.1 10.4 kidney 3.7, 9.7 10.8, spleen, 5.1 4.5 5.9 heart, 6.8, 3.3 testis, 7.9 11.2, skeletal muscle 3.4 4.8, small intestine, 1.0 4, pancreas 0.6 0.3 Human blood either defibrinated or oxalated, was inactive. Through the kindness of Dr H. Odum of the Department of Surgery three brain tumors were obtained immediately upon excision. Tested at pH 7, a positive result was obtained with an astrocytoma, negative results with a meningeal fibroblastoma and a glioblastoma.

*Precursor and Coenzyme* The production of *B* by brain during incubation was enhanced by the addition of extracts of brain or of other tissues. These extracts were prepared by grinding fresh (unincubated) tissue with sand in 5 per cent trichloroacetic acid, filtering or centrifuging and adjusting the pH to 6 before use. The effects were readily obtained with brain suspensions incubated at pH 7 as well as 6 and also in air. To double the rate of production of *B* without very much increasing the amount produced, brain suspensions were made up to contain a final concentration of 25 to 50 mgm. of brain extract per ml. and then incubated as usual. The total production was increased 50 per cent by using 5 times this amount of extract. Extracts of human blood and crude coenzyme II (15 per cent pure 1 mgm. per ml. of suspension) markedly increased both the rate and the amount of production. Extracts of rat liver kidney and skeletal muscle also increased the rate of production. We suppose that increases in rate of production due to the addition of extract signify the presence of a coenzyme, while increases in total amount indicate the addition of a precursor.

*Inhibitors.* In order to obtain some idea as to the possible physiological significance of the system producing *B*, the effect upon brain suspensions of a number of commonly employed drugs was examined. A preliminary survey was first undertaken in which very high concentrations were tested both for inhibition and acceleration. Since no drug was found to increase the rate by more than 25 per cent, this aspect of the problem was not investigated further. Likewise, those drugs which failed to inhibit by at least 50 per cent were also discarded. The tests were carried out as usual except that the pH was either 7 or 6.5.

The following is a list of drugs discarded in the preliminary survey

*Tested at 50 mgm. per 100 ml.* sodium azide iodoacetic acid nicotine acid ascorbic acid thiamin chloride riboflavin and pyridoxin *tested at 100 mgm. per 100 ml. of suspension.* chloral hydrate atropine caffeine cocaine antultrin dilantin dilaudid ephedrine guanidine histamine mecholyl metrazole nicotine phenobarbital pilocarpine papaverine picrotoxin physostigmine pitressin pitocin quinine strychnine salicylic acid agmatine adenine 2-aminopyrimidine acetylcholine digalton (Roche) digifolin (Ciba) dihydro-beta-erythroidin guanino hyoscyne L-histidine iodoacetic acid isocytosine iodoacetamide di-lysine ouabain phenylethylamine tryptophane quindine

Twelve agents were found which inhibited the production of *B* by more than 50 per cent. Aqueous extracts of digitalis leaves (Davies-Rose Upsher-Smith) inhibited by more than 90 per cent, but since the purified preparations listed above were without effect no further work in this direction was done. When

tested at 100 mgm per 100 ml, morphine inhibited 60 per cent,  $K_3Fe(CN)_6$  75 per cent, and benzedrine 90 per cent. The actions of morphine and benzedrine were not investigated further because close relatives of both appeared to act more powerfully.

In the case of the remaining 8 agents, the concentration was determined which inhibited the production of *B* by 50 per cent at pH 6.5 in oxygen, during a 50 minute period of incubation: 30 mgm per 100 ml of intercostrine, Squibb (purified curare), 0.005 M KCN, approximately 0.001 M ergonovine, 0.0001 ergotoxine, 0.00006 M ergotamine, 0.0001 M epinephrine, 0.0001 M vitamin K (2-methyl-1,4-naphthohydroquinone diacetate), 0.00001 M apomorphine, 0.0001 M emetine.

The action of cyanide may perhaps be explained on the basis of its ability to inhibit cellular respiration, though the relationship between cellular respiration and the production of *B* seems to be rather indirect. In the case of epinephrine, vitamin K, and apomorphine, a common mode of action may lie in their capacity to be reversibly oxidized and reduced, for each contains a benzene ring with two OH-groups, in the ortho position in apomorphine and adrenaline, and in the para position in vitamin K. Further support for this point of view lies in the fact that methylene blue at  $10^{-4}$  M inhibited the production of *B* by more than 90 per cent. The action of emetine and of the lysergic acid derivatives can not be explained. However, it is of interest that the ability of ergonovine, ergotoxin and ergotamine to inhibit the production of *B* parallels their toxicity.

*Chemical Structure of B* The data presented under *Methods* taken in conjunction with that below allow certain inferences to be drawn concerning the possible chemical structure of *B*. For the sake of brevity, we shall summarize it here in more or less outline fashion.

1 The colors produced from the reaction of thiobarbituric acid with sulfadiazine, or 2-aminopyrimidine, or *B*, are identical. This is strong evidence in favor of an aminopyrimidine structure. On the other hand, *B* is not identical with 2-aminopyrimidine, or with a derivative of the sulfadiazine type, because its reaction with thiobarbituric acid is more rapid, responds differently to pH, is blocked by nitrous acid, semicarbazide and hydrazine, and is subject to interference (or competition) by arylamines such as p-aminoacetophenone.

The nature of the absorption spectrum was discussed under *Methods*. The effect of pH upon color development in the thiobarbituric acid test is shown in figure 3, where it is seen that *B* behaves differently from 2-aminopyrimidine and sulfadiazine below pH 2.5. A further distinction is the time in the water bath required for maximal color development, which is less than 5 minutes for *B*, 45 minutes for sulfadiazine, and more than 135 minutes for 2-aminopyrimidine. The addition of 2 mgm of semicarbazide HCl or of hydrazine sulfate to the aliquot before adding the thiobarbituric acid reagent will inhibit subsequent color development by at least 90 per cent in the case of *B*, but does not affect the color from sulfadiazine. Preliminary treatment of brain extract with nitrous acid (0.5 ml of 1 per cent  $NaNO_2$  per ml of extract for 10 minutes, followed by 0.5 ml of 5 per cent ammonium sulfamate to destroy the excess nitrite) will inhibit subsequent color development by 90 per cent, whereas it will increase the color from sulfadiazine by 25 per cent, from 2-aminopyrimidine by 200 per cent, and from p-hydroxybenzaldehyde by 35 per cent (filter 400, not

heated) Interference by p-aminoacetophenone was described under *Parallelism Between Tests*

2 The possible presence of a characteristic oxidized group is suggested by several lines of evidence *B* is produced only under aerobic conditions. The arylamine test suggests an aldehyde (aromatic), and the test is blocked by semicarbazide and hydrazine which react with aldehydes and by thiobarbituric acid which reacts with aromatic aldehydes. On the other hand, the typical aromatic aldehydes reacted differently from *B* in the arylamine test and produced distinctly different and characteristic absorption spectra in the thiobarbituric acid test

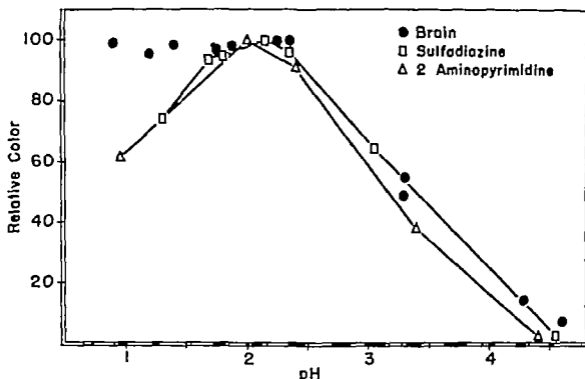


FIG 3 EFFECT OF pH ON COLOR INTENSITY IN THE THIOBARBITURIC ACID TEST

Note that 2-aminopyrimidine and sulfadiazine behave alike whereas brain extract (substance *B*) differs below pH 2

3 The fact that both tests interfere with each other as described under *Methods* and that both are blocked by hydrazine, semicarbazide, or preliminary treatment of the extract with nitrous acid, argues that both tests are concerned with the same molecule. However the behavior of both tests during the production of *B* and during its loss resulting from the ageing of extracts (see *Methods*) argues that each test is concerned with a separate locus on the molecule

In checking on the specificity of the arylamine test only certain aromatic aldehydes were found to produce color but the absorption spectra of none matched that of *B* the two closest being p-dimethylaminobenzaldehyde (peak at 450 millimicrons) and p-hydroxy benzaldehyde (peak at 370 millimicrons). The reactions of these two also differed from that of *B* in that less color was developed with p-aminoacetophenone than with p-aminobenzoic acid and in both cases the intensity of color was reduced by 50 per cent when the test was

carried out in 90 per cent alcohol. The intensity of color developed from *B* is not affected by these conditions. The addition of 2 mgm of either hydrazine sulfate or semicarbazide hydrochloride to the aliquots of extract inhibited subsequent color development in the arylamine test by more than 90 per cent, as did preliminary treatment of the extract with nitrous acid (as described above). It was noted under *Methods* that the absorption spectrum of the *B* thiobarbituric acid pigment is not that of an aldehyde.

4. The loss of activity following treatment of extract with nitrous acid suggests that an amino group is present in *B*. Diazotization, however, does not occur, because the addition of the Bratton and Marshall reagent *N*-(1-naphthyl) ethylenediamine after nitrous acid treatment produced no color.

#### SUMMARY

1. Suspensions of tissue incubated aerobically produce a substance arbitrarily called *B*, which can be measured quantitatively by two empirical tests. Evidence is presented suggesting that *B* contains an aminopyrimidine, an amino, and an aldehyde group.

2. The best producers of *B* are brain and liver, followed by kidney, spleen, heart, testis, and skeletal muscle. Small intestine, pancreas and blood produce none. One astrocytoma was active, whereas a glioblastoma and fibroblastoma were not.

3. A detailed study was made of the production of *B* by suspensions of brain, the chief results of which follow.

4. The addition of tissue extracts (blood, liver, kidney, etc.) to brain enhances the rate and amount of *B* production, suggesting the activity of coenzymes and precursors.

5. Apomorphine, ergotamine, emetine, ergotoxin, epinephrine, menadione, and ergonovine (arranged in descending order of potency) are the most powerful inhibitors of *B* production. KCN and methylene blue also inhibit.

6. The rate of *B* production is increased by incubation in oxygen instead of air, even though the rate of respiration remains unchanged. It is thus possible for oxygen tension to affect the metabolism of brain without changing the rate of respiration.

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# DINITROPHENOL CATARACT IN THE CHICK EFFECT OF AGE<sup>1</sup>

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In an excellent review of the literature upon the experimental and clinical studies relative to dinitrophenol Horner (1) stated that all attempts to produce cataracts by the administration of dinitrophenol in animals had failed.

We (2) reported in an earlier paper the production of cataracts in the young chick 10-50 days old. Inasmuch as all of the cataracts observed in man following the use of dinitrophenol had occurred in adults (30 years old or over), we thought it of interest to determine the influence of age upon the susceptibility of the lens of the chick to dinitrophenol

TABLE 1

*Effect of 2 4 dinitrophenol sodium 0.25% in the feed upon the lens and weight of chicks of varying age*

AGE WHEN PLACED ON DRUG DIET	WT. WHEN PLACED ON DRUG DIET	NO. OF CHICKS SHOWING GROSS OPACITIES OVER TOTAL NUMBER ON DRUG IN ANY GIVEN PERIOD DURING THE EXPERIMENTAL STUDY						TOTAL WEIGHT
		Day 1-5	Day 6-10	Day 11-15	Day 16-20	Day 21-25	Day 26-30	
Days	Grams							Grams
65	530	4/4	3/3	3/3	3/3	2/3	1/2	910
95	920	5/5	3/3	3/3	2/2	2/2		990
125	1350	1/2	2/2	2/2	0/1			1100
160	1875	1/2	2/2	1/1	0/1			1500
300	2240	1/2	0/1	0/1				2200

**PROCEDURE** All of the chicks used (white rock) were of a single hatching and were bought when one day old. They were kept on a diet of Purina-Startena until 4 weeks old and then placed on a growing mash which was later replaced by a laying mash. When the chicks were 65 days old or over as noted in Table 1, small groups were given a diet containing 0.25% dinitrophenol sodium. The chicks on the drug diet were observed daily for the presence of opacities in the lenses. At regular intervals chicks were sacrificed and the lenses removed for microscopical study.

**RESULTS.** In 14 out of 15 chicks varying in age from 65 to 300 days, the ingesting of food containing 0.25% dinitrophenol sodium led to the development of opacities of the lenses. In three chicks the opacities which developed during the first few days upon the drug diet cleared up later while the chicks were still on the drug diet. In one chick, 300 days old when placed on the drug diet no opacities were observed.

<sup>1</sup> This work was supported by a grant from the Mallinckrodt Chemical Works

Upon microscopical examination of sections from the lenses the typical changes as noted in our earlier study were observed, there were fine vacuoles in the lens fibers under the anterior capsular membrane and large destructive changes in the posterior portion of the lens. The lesions in the anterior portion of the lens usually healed in 6-10 days while the chicks were still on the drug-diet so that neither gross nor microscopic changes were observed. The lesions in the posterior part of the lens usually persisted until the chicks were sacrificed for microscopic study. In only one chick (300 days old) were there neither gross nor microscopic changes observed.

#### SUMMARY

The effect of age upon the development of cataract in chicks ingesting a diet containing dinitrophenol sodium has been investigated.

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# INFLUENCE OF TEMPERATURE, LIVER AND KIDNEY ON THE TOXICITY OF CAFFEINE IN *BUFO ARENARUM* HENSEL

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Caffeine is less toxic, per unit body weight in small toads than in large ones, in the former the weight of the organs and their metabolic functioning are proportionally greater. Further and contrary to what is usual with other drugs caffeine's toxicity is much higher at low temperatures and diminishes 'pari passu' with their increase (Günther and Odoriz 1945). Following these findings we have continued our studies and in the work here reported present explanations for the differences mentioned.

**METHODS.** We used in our experiments, carried out during the autumn, only males of *Bufo arenarum* Hensel, just gathered in order to obtain uniform results. For the experiments at different temperatures we placed the toads in glass containers with a definite quantity of water with the double purpose of avoiding dryness and maintaining a uniform temperature.

Caffeine sodium benzoate (Merck) in a 10% solution in Ringer was injected into the ventral lymphatic sac. To measure the amount of urine eliminated, during a period of time, the bladder was emptied and then the cloaca was occluded by means of a circular suture. In one series of experiments hepatectomy was carried out following Foglia's technique (Foglia, 1942). In an other series ligature of both ureters was practised by ventral paramedian incision under ether anesthesia. In some cases the amount of caffeine eliminated in the urine was determined.

**RESULTS** A) *Relation between temperature diuresis caffeine elimination and body weight.* To observe the influence of temperature on diuresis one group of toads was kept at 4°C and three others at 33°C during 90 minutes. In table 1 it can be seen that at 4°C the toxicity of caffeine is much higher than at 33°C the diuresis is about seventeen times smaller at the lower temperature. Another factor which influences diuresis is the weight of the body i.e. smaller animals have a greater elimination of urine per 100 grams of weight than larger ones. This is in agreement with the finding of Günther and de Soldati (1943) that the amount of renal parenchyma in *Bufo arenarum* Hensel is relatively greater in smaller toads.

In table 2 there is given in addition to figures on caffeine toxicity and urine excretion those of the percentage excretion of caffeine and xanthine derivatives (determination after Richter 1909) in a two hour period under high and low temperatures. The elimination of caffeine in 2 hours amounts to less than 10% of the injected quantity. The elimination of the xanthine derivatives in the controls was insignificant (0.15 mg/100 grams body weight).

To appreciate the effect of temperature alone on caffeine toxicity we used

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toads of the same weight Under this condition mortality is very high at low temperature (4°C) On the other hand, high temperature (33°-36°C) protects against the toxic effects of caffeine and only very slight symptoms of intoxication were observed

The elimination of urine in the controls placed at 36°C was approximately double that at 4°C The diuresis at 36°C is 50% greater in the caffeine group than in the control group But at low temperature not only is the diuretic effect of caffeine absent, but its toxic action at this temperature is so rapid that a suppression of urine flow occurs

TABLE 1

*Exp 17.IV.43 —Emptying of urine bladder, ligature of cloaca after a period of 30 minutes at the indicated temperature and then injection of caffeine sodium benzoate, 90 minutes later the urine was collected and measured*

GROUPS	INDIVIDUAL WEIGHT	EXPERIMENTAL TEMPERATURE	CAFFEINE DOSEAGE	MORTALITY OBSERVED WITHIN 90 MINUTES	TOTAL AMOUNT OF URINE ELIMINATED BY THE GROUP	QUANTITY OF URINE
	g	C	g/kg		cc	cc./100 g
Small	80-69 75-74 81-86 75	33	0.8	1/7	62	11.5
Large	148-154 169-150 165-139 150-158	33	0.8	1/8	124	10.0
Median	120-115 105-122 125-111	33	0.5	0/6	72	10.3
Median	114-110 120-102	4	0.5	1/6 later (4 dying with an intense hypertonus)	4	0.6

B) *Influence of temperature on toxicity* The protective action of higher temperature cannot be explained by the increased elimination of caffeine by the renal system because less than 10% of the drug is eliminated There must be other factors, i.e. a metabolic and a nervous one The metabolic acceleration due to higher temperature has the effect of increasing the chemical phenomena of detoxication (demethylation) Just a few degrees of temperature difference change the rate of metabolism Galli Mainini (personal communication) found in *Bufo arenarum* Hensel with 16°, 18.5° and 22°C the following metabolic rates 4.5-5.25-6.85 cc O<sub>2</sub>/100 g/hr in males at the end of winter In table 3 the remarkable diminution of mortality when the temperature goes from 11 to 25°C is shown

The striking difference of symptomatology observed cannot be explained by better elimination and detoxication at higher temperatures The symptoms

are markedly different right after the injection at a time when these two factors should have been unable to neutralize any great amount of active drug given. We must conclude then that this difference is caused by the special reactivities of the nervous tissue to this drug at different temperatures.

TABLE 2

Exp 27.IV.43 — *Bufo arenarum* Hensel males

Same experimental conditions as in previous table. Injection of 0.7 g./kg caffeine sodium benzoate. After two hours at the indicated temperature the animals were pitbed and the bladder urine collected.

GROUPS	INDIVIDUAL WEIGHT	TEMPERATURE	OBSERVED MORTALITY	URINE EXCRETION BY THE GROUP	URINE	ELIMINATION OF XANTHINE GROUP SUBSTANCES	TOTAL QUANTITY OF CAFFEINE ALKALOIDS INJECTED INTO THE GROUP	ELIMINATION OF CAFFEINE BY THE KIDNEY
	grams	°C.		g.	cc./100 grams	mg./100 grams	mg.	%
Large	156 150 157 145	36	0/4	67	11.0	1.73	162	6.5
Small	85 84 80	36	0/3	40	16.0	2.4	66	9.1
Median	110 105 100 104	36	0/4	61	14.6	1.5	111	5.7
	106 102 112 113	4	4/4	0	0	0	114	0
Controls	112 112	36	0/2	22.5	10.0	0.13	0	0
	111 110 116	4	0/3	15.0	4.45	0.15	0	0

C) *Influence of liver and kidney* It was very important to elucidate the role played by these organs in processes of caffeine detoxication. With the extirpation of liver or the ligation of both ureters at room temperature (20°C) there are no symptoms of a gross functional disorder within three days. The liver extirpation appears to be less injurious than the renal exclusion. The latter becomes still more injurious if the animal be placed at 33°C. The simultaneous exclusion of both organs has obviously a more severe effect than that of either one of them alone (see table 4).

TABLE 3

*Exp 20.III 43 and 2.IV 43 — Toxicity of 0.8 g/kg caffeine sodium benzoate at different temperatures*

The animals were placed for 20 minutes at the experimental temperature before the injection

GROUP	BODY SIZE	TEMPERATURE °C.	MORTALITY	SYMPTOMS
Controls	Medium	4	0/2	Hypotonus Total areflexia, except corneal reflex
	Medium	33	0/2	Slight hyperreflexia
Injected with caffeine	Medium 85-120g	4	2/2	Hypertonus very marked Slowing of all movements
	Medium 85-120g	33	1/10	Hyperreflexia Positional reflexes enormously accelerated
	Smaller than 80 g	11	3/9	Generalized muscular hypertonus Forelegs flexed and hindlegs extended
		25	0/9	Slightly excited

TABLE 4

*Exp 9.IV 43 — Effect of liver extirpation, ligature of ureters or both simultaneously at different temperatures*

NO OF TOADS	5	5	4	5
Mean weight, gm	100	120	>100	120
Operation	Total hepatectomy	Ligature of both ureters	Both operations	Ligature of both ureters
Temperature	20 C	20 C	20 C.	33 C
Symptomatology	Normal behavior for three days after	After three days some died, the others which survived at least five days were very edematous	Seventeen hours, one was normal, two with slowed positional reflexes and one with these reflexes abolished 20 hrs one dead Three days only one alive	Three and a half hours in good conditions All died before eighteen hours
Maximal survival time observed	> 3 days	5 days	3 days	< 18 hours

From table 5 it can be seen that the ligature of ureters in animals at 33°C, a procedure that by itself produces death in less than eighteen hours (see table 4), intensifies enormously the toxic effects of caffeine, i.e. in less than three hours

seven of the group of ten have already died the controls being all alive after this period

A dose of 500 mg/kg of caffeine sodium benzoate injected into a hepatectomized animal, not lethal in controls produces a definite mortality as can be seen in table 5. With a larger dose (800 mg/kg.) but smaller than  $DL_{50}$  the mortality is 100%. When both operations are carried out in the same animal, the dose of 200 mg/kg about a fifth of  $DL_{50}$ , shows a high mortality

TABLE 5

*Exp 31.III.43 and 18.IV.43 — Toxicity of caffeine sodium benzoate at different temperatures in animals with ligature of both ureters hepatectomy or both simultaneously*

TEMPERATURE °C	33	20	20	20	20	20	20
Operation	Ligature of ureters	Hepatectomy 3 hours before injection			Hepatectomy and Ligature of both ureters		
Dose mg./kg	800	500	800	1000	20	100	200
Number of animals	10	5	5	5	5	5	5
Indi. Ideal weight, g.	100-110-119 105-105-104 113-96-98 80	100-120	100-120	100-120	100-120	100-130	100-120
Symptoms tology	90 min all alive  120 min 6 dead and 3 dying  160 min 7 dead All died before 18 hrs	2 hours all alive	2 hours all dead	2 hrs 4 dead 1 dying	No symp- toms	No symp- toms	2 hrs 1 dead
Mortality observed 24 hours afterwards	10/10	2/5	5/5	5/5	0/5	0/5	4/5

\* All controls with caffeine and without ligature of ureters were alive 180 minutes after the injection

From the above experiments it can be deduced that the exclusion of the hepatic function does not disturb the mechanism of caffeine detoxication to such a degree as follows renal exclusion. It is very interesting to point out the important role played by the kidney in caffeine detoxication on account of the fact that the renal elimination of the drug as such is less than 10% of the injected dose.

There are two possible explanations for these results a) the renal tissue is able to detoxicate caffeine directly b) the kidney is able to eliminate the secondary products of caffeine or those produced by the intoxication itself

As Bock (1920) pointed out the toxicity of the secondary products of caffeine is much less than that of the original substance. That is why we believe that the first assumption is more probable, especially on account of the fact that the detoxication process is carried out in all tissues, with more or less intensity.

**DISCUSSION** In general, substances possessing a toxic action are more active when temperature is raised (Saint Hilaire, 1893, Langlois, Richet, 1895, Matisse, 1919, Lambruschini, 1938). But Sollmann (1942) describes experiments of Salant (1913) which prove that caffeine has a diminished toxicity at temperatures above 34°C. Saint Hilaire (1893) establishes that those substances which are eliminated quickly, increase their toxicity when temperature is lowered. This is the case of the drug under study: a higher temperature increases the renal function of poikilotherms as can be seen from the experiments here reported. At the same time caffeine accelerates its own elimination due to its diuretic effect, which was proved also directly in the frog's kidney by Richards and Schmidt, 1924, White, 1935, Hayman and Starr, 1925, and Richards, 1935.

The striking difference of toxicity between those drugs whose toxicity increases with the increase of temperature, and caffeine with a greater toxicity at low temperature, can be explained by the following two possibilities:

a) The interaction between tissues and drugs is intensified at higher temperatures in the case of most of the drugs. In the case of caffeine the demethylation process which is increased by higher temperature gives secondary products of low toxicity.

b) The higher temperature increases the excitability of the nervous system in a manner corresponding to the effect of the drug itself. It is interesting to note the existing parallelism between the actions of strychnine and cold in one side and the accelerating effect upon nervous reactions by heat and caffeine on the other.

The following data are derived mainly from studies carried out on isolated frog's nerve, which according to Gasser (1939) may be considered a sample of nervous tissues. The increase of temperature produces a shortening of chronaxia (Cr) and "Einschleichzeit" (EZ) and an increase of rheobase (Rb) (Schriever and Cebulla, 1939). The diminution of temperature increases Cr and EZ and diminishes Rb with rhythmic response to threshold current (Schriever and Ehrhardt, 1939). On the other hand, Schoepfle and Erlanger (1941), using single fibre preparations, found that cooling resulted in an increase of threshold and the height and area of the spike. We point out especially that Erlanger, Blair and Schoepfle (1941) showed that cooling increased threshold spontaneous oscillations. Strychnine produces an increase in Rb and also spontaneous oscillations (Erlanger, Blair and Schoepfle, 1941), a reduction of accommodation (Bartley, 1939) and of Cr (Lapicque, 1913). Cr is also reduced by caffeine (Flamm, 1929). The effect of cold on the central nervous system of batrachians was also demonstrated by Ozorio de Almeida (1943).

The findings mentioned above suggest the bases for understanding the greater susceptibility to strychnine of animals placed at high temperature<sup>2</sup> and that to caffeine of those kept at low temperature.

<sup>2</sup> Penhos (1943) found recently that  $DL_{50}$  of strychnine in *Bufo arenarum* Hensel at 5°C is about fifty times higher than at 35°C.

## SUMMARY

1) Per unit body weight, small *Bufo arenarum* Hensel compared with large ones, have a) more diuresis and b) after the injection of caffeine a larger amount of xanthine derivatives eliminated. In not a single instance was more than 10% of the injected caffeine eliminated in two hours

2) Toxicity of caffeine is much higher at low temperature (4 C) and diminishes with thermal increase

3) At low temperature symptomatology is more intense diuresis ceases and death comes earlier

4) Toxicity of caffeine is greater after certain operations and increases in the following order 1st hepatectomy 2nd ligation of both ureters 3rd with both operations together At 33°C ligation of ureters considerably increases the toxic action of caffeine.

We are indebted to Professor B. A. Houssay for his helpful advice and valuable criticism

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# THE ENHANCEMENT OF THE PHYSIOLOGICAL ECONOMY OF PENICILLIN IN DOGS BY THE SIMULTANEOUS ADMINISTRATION OF PARA-AMINOHIPPURIC ACID II<sup>1</sup>

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From reports appearing in the literature concerning penicillin it may be deduced that there are two outstanding obstacles to the extensive or intensive therapeutic use of the antibiotic agent granting its unusual efficacy. These problems are the preparation of penicillin and the wasteful manner in which the body disposes of such a valuable agent. The increasing availability of the agent attests to the strides which are being made in overcoming the first obstacle. The frequency with which large amounts of penicillin must be administered during the course of therapy is an acknowledgement of the second problem which has not been challenged adequately.

There appears, then, to be two means whereby one can make available more penicillin both from a practical and a therapeutic standpoint: 1) by increasing production and 2) by increasing the economy with which the body handles penicillin, from a physiological standpoint. The successful accomplishment of the latter problem is the subject of the investigations reported herein.

Three avenues are open for the excretion or inactivation of penicillin: 1) inactivation by oxidation, reduction, or conjugation within the body, 2) excretion of penicillin into the gastro-intestinal tract, either directly or indirectly, and 3) renal elimination.

Indirect but presumptive evidence has been pointed out for the inactivation of penicillin in the body when it is administered parenterally. Abraham, Florey, and associates (1), Rammelkamp and Keefer (2), and Rammelkamp and Bradley (3) found that 60 per cent or more of a single injection of 5,000 to 10,000 Oxford units of penicillin was recoverable in the urine. It was their opinion that the remainder probably was inactivated. Our investigations substantiate their findings and interpretation. However, we were not aware of information as to the probable mode of inactivation of the compound other than the work on "penicillinase" which has been reported to be a component or product of certain penicillin-resistant microorganisms (4).

It has been reported that penicillin administered parenterally appears in the bile in amounts which would suggest the process to be of only secondary importance (5), normally. A critical evaluation of the amount of penicillin ex-

<sup>1</sup> A preliminary note on this subject by Beyer, Woodward, Peters, Verwey and Mattis entitled "The Prolongation of Penicillin Retention in the Body by Means of Para Amino-hippuric Acid" was published in *Science*, 100: 107 (Aug. 4) 1944.

creted throughout the length of the gastro-intestinal tract would be complicated by the "enzymatic" inactivation of the agent by intestinal organisms (4)

The very rapid elimination of penicillin by the kidneys was first reported by Abraham Florey, et al and has been confirmed repeatedly. Indeed the remarkably large amount of penicillin that very rapidly appeared in the urine made it feasible to recover the agent for repeated use from the urine of patients receiving penicillin (1) when the amount of penicillin available was seriously limited by production facilities and methods.

The very great rapidity with which penicillin disappears from the blood stream and appears in urine is a major disadvantage in therapy and suggests that it might be eliminated by some other mechanism in addition to glomerular filtration. It occurred to us that it might be possible to suppress the secretion of penicillin by the simultaneous administration of p-aminohippuric acid (PAH)<sup>2</sup> which first was reported to be excreted by the renal tubular epithelium by Finkelstein, Aluminosa and Smith (6). Since then Smith and his associates have continued to study the renal excretion of PAH in humans and dogs (12) as has Bing (7), Lauson, Bradley and Courmand (8) and Warren Brannon and Merrill (9). Rammelkamp and Bradley have reported that the excretion of penicillin in urine was depressed by the injection of diodrast (3).

**METHODS** *The determination of penicillin in body fluids must necessarily be performed as a microbiological assay.* In this particular problem where the quantity of penicillin in plasma and urine was to be determined, the usual Florey cup plate method was not completely satisfactory because of the small concentration of penicillin that frequently occurred in these body fluids at various points in the experiment. Therefore we used a modification of the technique that was proposed by Rammelkamp (10). He made use of the fact that penicillin in extremely small quantities inhibited the growth and, correspondingly, the hemolysin production of  $\alpha$  hemolytic streptococci.

In our experiments, as in his, 0.2 cc. of various dilutions of body fluids containing penicillin was added to 0.5 cc. of a one percent horse blood broth containing a 1:1000 dilution of an 18-20 hour Group A streptococcus culture. If there was sufficient penicillin in the dilution to inhibit the growth of the streptococcus the culture, after incubation, was clear and the unhemolyzed red blood cells had settled to the bottom of the tube to form a readily recognizable button. If on the other hand there was insufficient penicillin in the tube, the streptococcus culture on incubation grew and produced hemolysin which in turn lysed the red blood cells. Such tubes could be differentiated readily from those in which hemolysis had not occurred. All assays were incubated at 37°C for 18-22 hours.

Tests with known concentrations of penicillin indicated that the streptococcus culture used was sensitive to 0.005 to 0.002 Oxford Unit of penicillin. Inasmuch as 0.2 cc. of body fluid or a dilution thereof was employed in the test, 0.01 or 0.02 unit of penicillin per cc. of plasma or urine could be detected. A few of the

<sup>2</sup> Since this article was submitted for publication it has been decided to use the abbreviation (PAH) for p-aminohippuric acid and its sodium salt instead of (PAHA) which appeared in the original report and also in the figures submitted with this manuscript.

earlier experiments were performed using double dilutions for the titration of penicillin in body fluids. It soon became apparent that the increments between dilutions could be cut in half without sacrificing the accuracy and sharpness of the endpoint. Consequently, the majority of penicillin titrations have been performed using a half step dilution system which progressed 1 2, 1 4, 1 6, 1 8, 1 12, etc. This procedure was very valuable in that it doubled the accuracy with which individual penicillin values could be determined and made it possible to obtain much more reliable and critical figures for clearance values, percentage recoveries, plasma concentrations, etc. A standard penicillin solution was always included in each group of penicillin titrations and the unitage of various samples was assigned on the basis of a comparison with the standard solution.

Wherever possible, the quantity of penicillin used was checked by means of the standard Florey cup plate procedure. All values for the dosage of penicillin administered and the percentage of penicillin recovered in the urine were deter-

TABLE 1  
*Recoveries of penicillin from urine of dogs receiving PAH*

PENICILLIN SODIUM  Experiment	2,460 OXFORD u/cc.  pH	1 cc. DILUTED TO 50 cc. = 49.2 OXFORD u/cc.  Duplicate determinations	
		Sample 1	Sample 2
I Distilled H <sub>2</sub> O (Control)	6.8	52.5 u/cc	52.5 u/cc
II Urine	7.1	45.0 u/cc	45.0 u/cc
III PAH urine (conc PAH = 559.4 mg / 100 cc)	4.5	47.7 u/cc	47.7 u/cc

mined by means of the Florey cup plate procedure, which is conceded to have an accuracy of about  $\pm 15$  per cent.

There were a number of factors that we felt might influence our results, and these were eliminated as sources of error. In table 1 are compiled data concerning the effect of adding penicillin to normal urine at essentially neutral pH and to urine containing a high concentration of PAH at pH 4.5, the samples being obtained from a dog. Equal amounts of penicillin were added to the urine samples and to water, and were allowed to stand at room temperature for 4 hours, a period exceeding considerably the maximal length of time urine or plasma samples were permitted to stand before refrigeration at 0-5°C. The results presented in table 1 show that there was no significant decrease in the penicillin content of urine under these conditions, as assayed by the Florey cup plate method.

To use the more sensitive Rammelkamp method of assaying penicillin it was necessary to conduct all the experiments under aseptic conditions, a situation new to renal clearance studies in dogs, so far as we were aware. This necessitated designing apparatus for the collection of urine and washing out the bladder with

air and water which made use of a closed easily sterilized system of bottles tubes and filters as diagrammed in figure 1. The apparatus has proved most satisfactory for the collection of sterile urine samples.

The procedure for the use of this apparatus for the collection of urine samples was as follows. Clamp (a) was released the urine was permitted to flow by gravity into the sterile collecting bottle (A) and the bladder was gently compressed by pressing on the abdomen. Clamp (a) then was closed and clamp (b) was opened to permit inflation of the bladder by blowing through the sterile filter (B). Next, clamp (b) was closed and (a) was opened to exhaust all urine

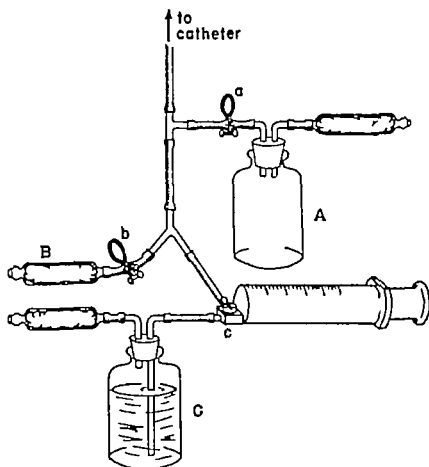


FIG. 1 A DIAGRAMMATIC REPRESENTATION OF THE APPARATUS USED TO COLLECT URINE AND WASH OUT THE BLADDER OF DOGS ASEPTICALLY IN THE COURSE OF THE REMAL CLEARANCES

from the bladder into the bottle (A). Clamp (a) was replaced and the 3 way valve (c) was turned to permit drawing 15 cc. of sterile distilled water from bottle (C) into the syringe. The valve (c) then was turned to permit the injection of water into the bladder. Valve (c) then was closed, clamp (a) was opened, and the water was allowed to drain from the bladder into the urine sample. Following this the bladder again was washed with air. All transferring of bottles and samples was carried out using the usual bacteriological precautions and the samples were refrigerated at 5 C until the assays were performed. Blood was drawn aseptically into syringes containing sterile sodium citrate, appropriate

volume corrections being applied both here and for the washout dilutions of the urine samples

Our procedure varied somewhat with the nature of the experiment, but in general it has been as follows. The female dogs were given 40 cc of water/kgm of body weight by stomach tube, the animals were then placed on an electrically warmed padded table, and lightly restrained. The perineum, tail, and thighs were clipped and then shaved. The whole of this area was washed with soap and water and then, with a vaginal swab of 'ST-37' hexylresorcinol antiseptic solution in place, the whole of the shaved area was swabbed twice with a mercurial antiseptic. The dog then was fitted with sterile drapes and, without otherwise touching the animal, the vaginal swab was removed and a sterile speculum was inserted into the vagina. The bladder was catheterized through the urethra, using a sterile lubricated catheter and guide. The catheter then was connected with the urine collecting apparatus, and the bladder was drained, washed with air, and then washed twice with 15 cc of 'ST-37' hexylresorcinol antiseptic solution. After 2 to 3 minutes the solution was drained from the bladder, which was washed with 3 or 4 successive 15 cc washings of distilled water.

Immediately before injecting penicillin intravenously, the urinary bladder was emptied, washed with air, then 15 cc of water, followed by air. This total sample was always saved. A pour plate was made from this to check for bacterial contamination and a blank penicillin assay was performed to make certain that the 'ST-37' hexylresorcinol antiseptic solution was completely removed from the bladder. Close attention to these details abolished for practical purposes the appearance of contamination sufficient to interfere with the test.

The procedure for the determination of p aminohippuric acid was similar to the Bratton and Marshall (11) method for the determination of sulfonamides in plasma and urine. We have modified the method as it appears in the monograph by Goldring and Chasis (12) to suit our particular needs.

The very fact that PAH can be so easily, rapidly, and conveniently determined in blood and urine makes its use in conjunction with penicillin therapy far superior to any other known agent that might be found suitable for this purpose, even if another agent should be found to be equally satisfactory in all other respects. The reagents necessary for these determinations are to be found in the laboratories of almost all hospitals where sulfonamide determinations are performed routinely. During all these experiments PAH was administered intravenously, the priming dose, rate, and concentration of the solution being determined by the nature of the experiment and the blood level desired. Other details of the procedure will be given with the description and results of the experiments.

**EXPERIMENTAL** *Experiments lasting two hours* were performed for the purpose of orientation and to determine in unanesthetized dogs the validity of our hypothesis that there might exist a competition between penicillin and PAH for the same renal excretory mechanism. In these experiments the sodium salt of p aminohippuric acid, buffered at pH 7.4, was used. We customarily injected a priming dose of 120 mgm/kgm of PAH and followed it with a continuous infusion of a 2.25 per cent solution at a rate of 3 cc/min. These conditions were

altered slightly to give plasma concentrations of 30 to 50 mgm./100 cc. Customarily the plasma PAH concentration was permitted to equilibrate over a period of 15 minutes and then 2 or 3 successive 10 minute clearance measurements were performed. Creatinine clearances were performed on these dogs to determine the constancy of their glomerular filtration rate.

After the introductory clearances were finished 10 000 Oxford units of penicillin were injected intravenously and alternate ten minute clearances were carried out for the duration of the experiment. All the urine was collected and the penicillin content of each specimen was determined by the modified Rammelkamp method. From these data the periodic cumulative recovery was calculated as a percentage of the amount injected. All the urine specimens finally were pooled and aliquots of the pooled urine were assayed by the Florey cup plate method as a check on the other determinations. Control experiments were carried out wherein 10 000 oxford units of penicillin were injected but no PAH was administered. One dog was given 7 grams of sodium bicarbonate with the priming dose of water to maintain alkaline urine in the PAH and corresponding control experiments.

Since the results of the 2 hour experiments served to substantiate our hypothesis and to guide our subsequent work and since they were the least complex, they have been presented here in some detail. Data pertaining to the clearance tubular excretory mass ( $T_m$ ) etc for PAH have been reserved for the detailed presentation of the pharmacology of that compound to be published separately thus simplifying the present report.

Figures 2 and 3 summarize several experiments on two dogs concerning the renal clearance of penicillin at falling plasma levels during the maintenance of a constant plasma level of PAH and when no PAH was given. Although the results were essentially similar some differences in the two figures justify the inclusion of both.

In both figures the upper straight line was drawn through the trend of the data of the normal clearances of penicillin at falling plasma concentrations. No attempt was made by us to determine the renal tubular mass having to do with the excretion of penicillin. It may be noted however that the clearance of this material was extremely high. These values of over 200 are similar to those considered normal for the measurement of minimal renal plasma flow. In each figure the average creatinine clearance for the dog is given so that one may compare the penicillin clearances with the known glomerular filtration rate for the animal. This served further to demonstrate that penicillin was excreted largely by way of the tubular epithelium. It was found in the course of the longer experiments that neither PAH nor penicillin influenced creatinine clearance.

Ideally if PAH competed with penicillin for the same tubular excretory mechanism, it should have been possible to suppress the clearance of the latter compound to that representing glomerular filtration by increasing the concentration of PAH in plasma to the point where it exceeded the maximal tubular excretory mass for the compound. However other factors appeared to complicate the picture, since at the higher plasma concentration of penicillin PAH suppressed its clearance to below the glomerular filtration rate, figures 2 and 3.

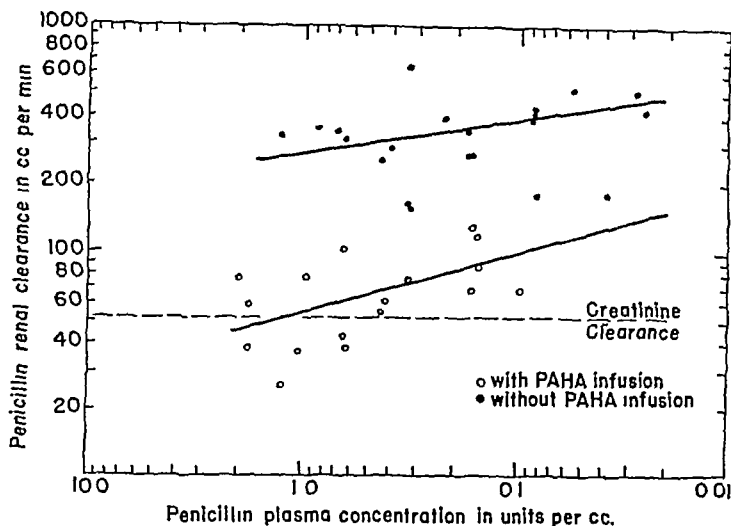


FIG 2 Dog 84, Wt = 15 Kg

The relationship of plasma concentration to the renal clearance of penicillin, normally and as influenced by sodium p-aminohippurate, the plasma concentration of which was 45 mgms/100 cc. Actually, two to three separate experiments are presented by the coordinates for each curve

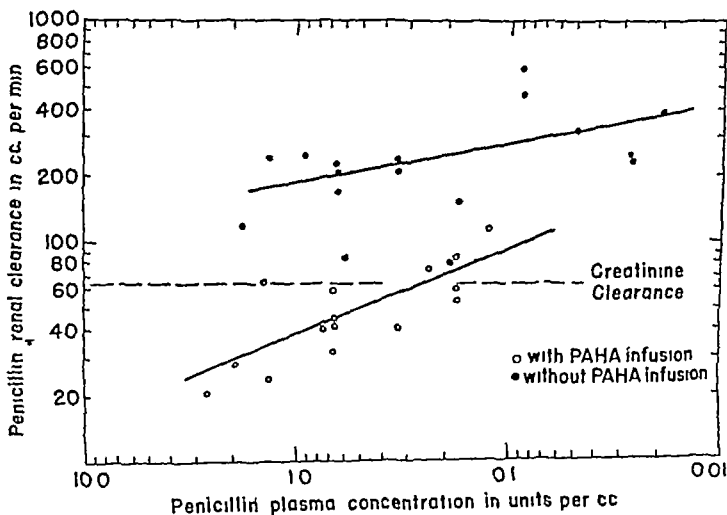


FIG 3 Dog 32, Wt = 18.4 Kg

The relationship of plasma concentration to the renal clearance of penicillin, normally and as influenced by sodium p-aminohippurate, the plasma concentration of which was 30 mgm/100 cc. Actually, two to three separate experiments are represented by the coordinates for each curve

This was most evident in the case of Dog 32 and may mean that some of the penicillin was bound by plasma proteins that there was a back diffusion of penicillin, or that it was both excreted and reabsorbed by the renal tubules. Because of the magnitude of the inherent error in the microbiological assay of penicillin really reliable figures for its binding on plasma proteins are not obtainable at present.

It should follow from the decreased clearance of penicillin when PAH is administered simultaneously that the blood level subsequent to the injection of a single dose of penicillin should be maintained at higher levels longer than in the control penicillin experiments. Figure 4 illustrates a single experiment which

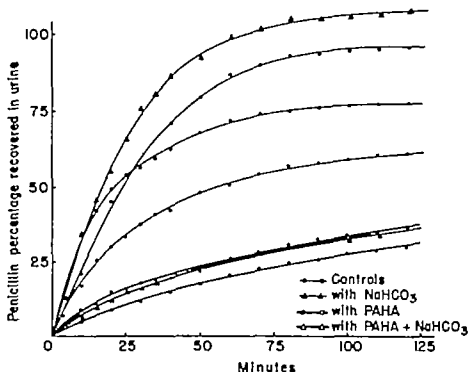


FIG. 4. DOG 84. THE PROGRESSIVE AND TOTAL RECOVERY OF PENICILLIN FROM URINE PER UNIT TIME FOLLOWING THE INTRAVENOUS INJECTION OF 100 000 OXFORD UNITS

The upper four curves represent the control values and the lower three curves represent the effect of sodium p-aminobiphenylate on the recovery of penicillin from urine. When sodium bicarbonate was administered the pH of the urine was 7.8 to 8.0 whereas in the other experiments the pH was 4.5 or above.

typifies these results. The graphical presentation of these data best illustrates the rapidity with which penicillin is normally lost from the blood stream. Within less than 15 minutes following the administration of 10,000 units of penicillin intravenously to a 15 kg. dog less than 10 unit/cc. remains in the plasma, normally. This initial decrease is probably a reflection of the very rapid dissemination of the antibiotic agent into the tissue in addition to the excretion of a very large amount of the material. Following this initial phase the plasma concentration decreases more slowly, but even so only a trace of penicillin remains in the plasma 90 minutes following the injection.

When PAH is administered continuously before and following the injection of

penicillin, the initial rapid fall is inhibited, but probably only to the extent that the excretion of the compound is decreased. Curves and data such as are presented in figure 3 demonstrate that, in keeping with the decreased clearance of penicillin, its plasma concentration is maintained at a much higher level over a 2-hour period in the presence of PAH than in its absence. However, from these data it is difficult to evaluate the efficacy with which PAH causes a retention of penicillin by the body.

Normally, 35-75 per cent of the amount of penicillin injected may appear in the urine within the first 30 minutes following its administration. On the other

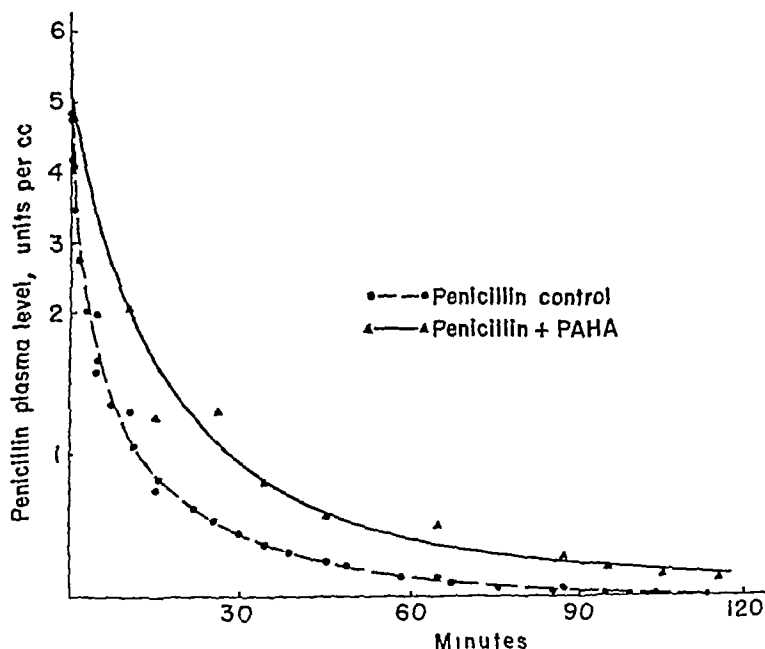


FIG 5 PENICILLIN PLASMA LEVELS FOLLOWING A SINGLE INTRAVENOUS INJECTION OF 10,000 OXFORD UNITS, NORMALLY AND AS INFLUENCED BY THE CONTINUOUS INFUSION OF SODIUM p-AMINOHIPPURATE TO MAINTAIN A PLASMA CONCENTRATION OF 30 MGM/100 CC

hand, when PAH was administered intravenously to maintain plasma concentrations of about 30-40 mg/100 cc the recovery of penicillin in the urine at that time ranged from 12 to 18 per cent of the amount injected. In figure 5 are presented curves representing the progressive recovery of penicillin in urine during the control experiments and when PAH was infused. These curves present a striking reduction in the rate and over-all recovery of penicillin when PAH was infused as compared with the control experiments wherein PAH was omitted.

Ordinarily, the urine of dogs administered PAH is acid and may reach a pH of

4.5 Recognizing the effect of pH on the stability of penicillin experiments essentially similar to the above were performed wherein 7 grams of sodium bicarbonate were added to the usual 40 cc./kg. priming dose of water given by stomach tube to a dog in order to produce a urinary pH between 7 and 8.

Curves representing the percentage recovery of penicillin from the alkaline urine of this dog with and without the intravenous infusion of PAH are also presented in figure 5. It may be seen that alkalization of the urine did not influence

TABLE 2

Dog 39 18.4 kg

An abbreviated protocol of penicillin control and PAH infusion experiments on an unanesthetized normal dog. A single 10,000 unit dose of penicillin was injected intravenously after a 15 minute period for the stabilization of the intravenous PAH infusion.

PENICILLIN CONTROL					PENICILLIN PLUS PAH INFUSION						
Time	Urine flow	Plasma conc.	Penicillin clearance	Progressive recovery	Urine flow	Plasma conc.	Penicillin clearance	Progressive recovery	PAH plasma conc.	PAH clearance	Time
hr./min.	cc./mi.	units/cc.		Per cent	cc./min.	units/min.		Per cent	mg./100 cc.		hr./min.
- 10					7.2				30.0	95.3	- 10
00					6.83				28.3	91.0	00
01		2.67				2.74					02
05		0.90				1.28					03
10	5.74	0.90	345.7	26.5	6.54	1.20	57.00	9.5	28.1	89.6	14
15		0.47				1.28					20
20	4.37		247.4	38.6	6.00		36.98	14.6	27.7	92.3	26
25		0.23				0.66					31
30	5.05		396.6	47.3	6.10		37.50	16.8	27.7	98.0	36
35		0.34				0.43					41
40	4.90		151.7	52.1	6.32		59.97	19.0			46.5
45		0.17				0.44					50.5
50	5.60		272.8	56.6	5.67		54.38	20.0	27.1	98.3	55.5
60.5				61.0				22.3			1.04
1.05		0.09				0.16					1.09
1.10	4.80		438.6	64.7	4.27		114.76	24.1	24.6	96.2	1.15
1.20				68.2		0.16					1.20
1.25		0.06			5.10		82.88	25.3	27.4	90.5	1.25
1.30	4.20		413.4	71.7							

the marked reduction in the recovery of penicillin when PAH was infused during the experiments.

To summarize the method and results of the 2-hour experiments table 2 has been included. This table presents abbreviated data for approximately the first 90 minutes of two successive experiments using another dog. The first experiment was a penicillin control and the second incorporated the continuous infusion of PAH. An interval of one week occurred between experiments. It may be noted that initially the plasma concentration of penicillin was approximately the same in both cases but decreased much more rapidly in the control experiment. The penicillin clearances in the control test were of the order of the nor

mal renal plasma flow of a dog of this weight. Unavoidably they vary more than the PAH clearances because of the limitations of accuracy of the penicillin assay, which may exaggerate errors inherent in this procedure. When PAH was infused, the clearance of penicillin was suppressed to below the normal glomerular filtration rate of this dog (62.2 cc/min). This marked suppression of penicillin clearance was accomplished at a PAH plasma concentration of approximately 28 mg/100 cc. It is apparent from the clearance values that the concentration of PAH brought to the renal tubules is in excess of their ability to secrete all of it for the values are much less than the maximal clearance of PAH or penicillin at very low plasma levels. The urine flow maintained throughout both experiments is more than adequate.

*Twelve-hour experiments* were carried out wherein a given plasma level of PAH was established, a single priming dose of penicillin was administered intravenously, and the plasma PAH level was maintained for the duration of the experiment. Control 12 hour experiments also were performed during which a single injection of penicillin was made but no PAH was administered. In detail these experiments were much like the earlier ones except that it was advantageous to administer a barbiturate to maintain the dogs in an hypnotic state. The purposes of this work were to ascertain how long a determinable concentration of penicillin was maintained in plasma and urine and to estimate the recovery of penicillin in urine normally and in the presence of PAH. These experiments also served for orientation purposes for subsequent work.

We have used the two-hour experiments to substantiate our hypothesis. Since these results merely confirmed and extended the initial findings of the two-hour experiments they have been summarized in table 3. By doing this, interesting, but voluminous, data have been omitted. All the experiments are presented in the table.

It may be concluded from the data presented in table 3 that the salient point in our hypothesis and the results of the two hour experiments on unanesthetized dogs were substantiated by these longer experiments. Certain individual values that were at variance with the trend of the data in the experiments do not influence the significance of the over-all results, since there were so many cross checks in these data. These experiments may be summarized as demonstrating the following points:

1. There was invariably a slower rate of fall in the penicillin plasma level after the single 10,000 unit intravenous injections when PAH was infused during the course of the experiment. This may be judged by comparing the five-minute and two hour penicillin plasma levels in the experiments on a single dog.

2. The time at which the penicillin level in the plasma fell below determinable concentrations was prolonged from 2 to 2½ hours for the controls (except in one instance) to from 3½ to 4 hours in the PAH experiments. This finding should have been obtained if the first conclusion were true.

3. There was a prolongation of the duration of time wherein penicillin was recoverable in the urine from 6½ to 8 hours for the controls to from 9½ to over 12½ hours for the PAH experiments.

4 Since the rate of fall of the penicillin plasma level was decreased and its time of disappearance from plasma and urine was increased, it was consistent that we should find the amount of penicillin recovered in the urine especially during the early hours of the experiments, to be very strikingly less in the PAH tests as compared to the controls

5 The data presented in table 3 for the renal clearance of penicillin in the two types of experiments clearly showed that PAH suppressed the clearance of penicillin and that this suppression was more or less proportional to the decreased PAH clearance as the plasma concentration of the latter compound was increased

TABLE 3

*A condensation of the data obtained on the three dogs used in the experiments of 12 hour duration*

CRITICAL POINTS FOR COMP. SHOW	dog 155 12.5 kg.		dog 53 19.4 kg.				dog N 22.3 kg.	
	Control glucose-saline infused L	PAH infused L	Control #1 glucose-saline infused Lv	PAH #1 infused L	Control #2 glucose-saline infused L	PAH #2 infused L	Control glucose-saline infused L	PAH infused Lv
Penicillin plasma level 5 mins after injection units/cc	2.92	2.61	3.06	0.69	2.00	2.40	0.04	1.76
Penicillin plasma level 2 hrs after injection units/cc	0.02	0.16	0.18	0.06	0.00	0.10	0.06	0.33
Penicillin absent from plasma at hrs mins	2.30	3.30	3.30	3.33	2.00	3.31	2.31	3.50
Penicillin absent from urine at hrs mins	6.35	>12.35	6.54	11.10	7.04	9.25	8.05	11.30
Penicillin av clearance during maintenance of plasma level cc/min	207.9	61.7	212.8	96.8	181.3	130.4	170.9	71.6
Penicillin percentage recovery in urine during 1st hour of experiment	84.6	19.7	77.0	17.6	79.2	48.8	35.1	22.8
Urine flow av cc/min	2.8	2.5	2.2	3.3	2.2	2.7	0.8	1.51
PAH av plasma level mg./100 cc		48.1		41.5		29.6		46.7
PAH av clearance cc./min		74.7		114.9		156.8		99.6

This interference by PAH with the tubular excretion of penicillin was the basis for the interpretation of the findings listed from 1 to 4

A number of other correlations can be made between the various data if one studies the table carefully. To enumerate them would only burden the evidence for our conclusions.

**DISCUSSION** This research was begun at a time when the supply of penicillin was critically low with an idea of increasing with the aid of PAH the amount of penicillin available for treatment by increasing the physiological economy of the agent and thus decreasing the amount needed to treat a given case. For this reason the experiments were designed to determine whether PAH reduced the excretion of penicillin as judged by the prolonged maintenance of high plasma

concentrations, prolongation of the period over which the drug was excreted, reduction in the rate of excretion, and the marked decrease in the amount recovered in urine in a given period of time

From a physiological standpoint, the observation that the renal clearance of penicillin is normally of a magnitude which suggests that it probably is an index of renal plasma flow even at what may be considered greater than usual therapeutic levels is a point of considerable interest. That penicillin is excreted by the renal tubular epithelium in addition to glomerular filtration seems certain, since its clearance can be suppressed even to or below glomerular filtration rate by the administration of PAH, which is used to measure both minimal renal plasma flow and tubular excretory mass (12)

It should be pointed out that in this case we have probably established therapeutic as well as physiological economy of penicillin, for the assay depends on the measurement of the bacteriostatic activity of penicillin-containing plasma removed from the animals. Other evaluations of this point will be made clinically

The pharmacology of p-aminohippuric acid has been omitted in order to simplify and shorten this presentation. Detailed experimentation will be reported in another article showing that sodium p-aminohippurate has a very low order of toxicity (less than that of sodium hippurate), either acutely or following prolonged administration, and that it is most suitably administered intravenously in order to maintain high plasma concentrations

#### SUMMARY

It has been found that the continuous infusion of sodium p-aminohippurate facilitates a physiological economy by the body of single intravenous injections of penicillin. When compared with similar experiments, wherein penicillin but no p-aminohippuric acid was injected, the effectiveness of the latter agent may be judged by 1) the prolonged maintenance of a determinable plasma concentration of penicillin, 2) a marked prolongation of the period of time over which penicillin was excreted, 3) a definite decrease in the rate of excretion of penicillin, and 4) a marked decrease in the amount of penicillin recovered in the urine in a given period of time

It was concluded that the manner in which this effect has been produced was by a competition between p-aminohippuric acid and penicillin for excretion by the renal tubular epithelium. This appeared to be the case, since the renal clearance of penicillin, which normally approximates the renal plasma flow of dogs, can be depressed to or below that measuring glomerular filtration rate by the simultaneous administration of sodium p-aminohippurate intravenously to maintain a plasma concentration that causes its own clearance to be less than maximal

*Acknowledgements* Since to conduct properly research of this type the very closest cooperation among a large staff of investigators is essential, it is impossible to give everyone the credit he deserves as one of the list of authors. However mention should be made of the work of Miss Elizabeth A. Patch and her associates who developed the PAH procedures used in these investigations

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# N<sub>1</sub>-DIMETHYLACROYL SULPHANILAMIDE (IRGAMID)

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N<sub>1</sub>-dimethylacroyl sulphanilamide (Irgamid) is fairly closely related to sulphacetamide in structure and shares with it the advantage that the solution of its sodium salt is considerably less alkaline (pH 8.4) than that of the other sulphonamides. Experiments with rabbits suggested that Irgamid is easily absorbed and the concentrations obtained in the blood are considerably higher than those reached after similar doses of sulphapyridine, (Högger, 1941). Swiss workers (Markoff, 1941 and 1942, Scherer, 1942) have reported favourably on effect of irgamid in various types of infections and it was thought worth while to introduce a small supply of the drug for trial in this country. It is unlikely, however, that irgamid will be freely available during the war. Indeed in its country of origin it seems to be being displaced by N<sub>1</sub>-(3,4-dimethylbenzoyl)-sulphanilamide (Irgafen). The present investigation must therefore be regarded as chiefly of academic interest. It was undertaken to determine the blood concentrations produced and tolerated in human subjects after administration of the drug by various routes, rather than as a study of the therapeutic effects.

**METHODS** The method used to determine irgamid was essentially that of Bratton and Marshall (1939). Preliminary estimations of irgamid added to blood showed that an initial dilution of 1 in 20 gave complete recovery up to concentrations of 4 mg per cent. At higher concentrations a 1/40 dilution was necessary for complete recovery and even this was not adequate for concentrations greater than 16 mg per cent. We have used a 1/40 dilution throughout, applying a correction for apparent concentrations greater than 16 mg per cent. Pulver (1941) has made similar observations on irgamid recovery and suggests that an easily hydrolysed haemoglobin-irgamid complex is present in blood. We have added 0.5 cc blood to 17.5 cc water, allowed the mixture to stand for 5 min and then added 2 cc 20 per cent trichloroacetic acid. The mixture was centrifuged rather than filtered, since filtration gave irregular values—probably due to absorption on the filter paper. Duplicate 5 cc samples of supernatant fluid were pipetted off for analysis.

There was no difficulty in estimating irgamid added to urine.

**ADMINISTRATION** To study the rate of elimination, six patients with no gross organic disease were given a single intravenous dose of sodium irgamid (2 or 3 g), two patients were given a single intramuscular dose of 3 g. Blood samples were collected at frequent intervals over a 48 hour period, and in most cases urine was also collected and analysed.

To compare the concentrations in the blood and the spinal fluid, five patients were given 2 g sodium irgamid intravenously, together with 1.5 g by mouth, i.e.

the priming dose for severe infections. Four hours later specimens of blood and cerebrospinal fluid were obtained for analysis.

Eleven patients with various types of infection were given repeated doses of irgamid according to the schedule recommended by the Medical Research Council (1943). Five of these patients had the course for mild infections, i.e. an initial dose of 2 g. by mouth followed by 1 g. 4-hourly for 48 hours, 1 g. 6-hourly for a further 48 hours and finally, 0.5 g. 6-hourly for 48 hours. The larger doses for severe infections were given to 6 patients, the full course being 2 g. intravenously together with 1.5 g. by mouth followed by 1.5 g. 4-hourly for 2-3 days, 1 g. 4-hourly for 2-3 days and finally 1 g. 6-hourly for another 2 days. Two children were treated with irgamid for a relapse of influenzal meningitis and two others for bronchopneumonia. One patient was given repeated intramuscular injections.

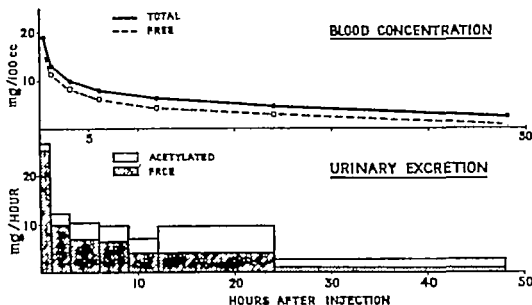


FIG. 1. BLOOD CONCENTRATION AND URINARY EXCRETION OF TOTAL AND FREE IRGAMID AFTER A SINGLE INTRAVENOUS INJECTION OF 3 G. OF THE SODIUM SALT.

It was thought unnecessary to dilute the drug for intravenous or intramuscular administration. 10 to 15 cc. of a 20 per cent solution were injected intravenously over 3 minutes without ill effect. The intramuscular injections were given into the outer side of the thigh. Each was accompanied by considerable pain which persisted for 15 to 20 minutes. It seems likely that this discomfort was partly due to the bulk of fluid injected. There was no apparent local reaction, but in a patient with tuberculous meningitis who had two intramuscular injections, some evidence of tissue damage was found at autopsy.

**RESULTS.** A typical curve obtained by estimation of total and free irgamid in blood after a single intravenous dose of 3 g. is shown in figure 1. The proportion of acetylated form increases with time and although acetylation proceeds to various extents in different individuals, it was found in general that the acetyl compound represented 20 per cent of total after 3 hours and 50 per cent after 24 hours. In fact the actual amount of the acetyl compound present seemed to be

practically constant after the first 3 hours. Comparing these results with those of Strauss, Lowell, Taylor and Finland (1941), it appears that irgamid is less readily acetylated than sulphapyridine and sulphanilamide, but rather more readily than sulphathiazole. It was also found that when given intravenously irgamid is relatively rapidly excreted, 80 per cent of the total urinary excretion occurring in the first 24 hours as compared with 70 per cent in the case of sulphanilamide and sulphapyridine and 90 per cent with sulphathiazole. The amount of acetyl form present in the urine varied, and showed a general tendency to increase, reaching a maximum of 80 per cent of total during the second 24 hour period after injection.

After a single intramuscular injection of 3 g in two patients, maximum blood concentrations of 11 and 12 mg per 100 cc were reached at the end of an hour, and the level had fallen by half after 12 hours. By giving repeated intra-

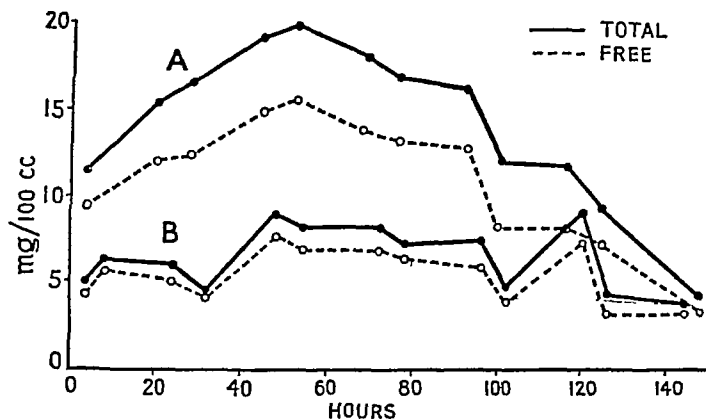


FIG 2 BLOOD CONCENTRATION OF TOTAL AND FREE IRGAMID DURING ADMINISTRATION OF  
A COURSE FOR SEVERE INFECTIONS B COURSE FOR MILD INFECTIONS

muscular doses of 1 g 12 hourly it was possible to maintain a fairly constant blood level of 6 to 8 mg per 100 cc.

In the series of specimens obtained 4 hours after administration of an intravenous plus an oral dose of irgamid, the concentration in the cerebrospinal fluid averaged 50 per cent of that in the blood. Daily lumbar puncture on one patient receiving repeated doses by mouth showed that the concentration in the cerebrospinal fluid gradually increased, and for a short time after discontinuing the drug it remained at a considerably higher level than the blood concentration. Irgamid was determined in a specimen of ascitic fluid from a patient with subacute hepatitis and the concentration found to be about 75 per cent of that in the blood. The drug seems to be about equally distributed between cells and plasma—the concentration in the red cells tending to be slightly higher than that in the plasma.

Blood concentration curves obtained during repeated large and small doses are

shown in figure 2. The maximum concentrations attained showed considerable variation (see table 2) but were consistently higher than those reported by other workers after similar doses of sulphapyridine. In one patient (No. 11) given a course of 15.5 g. by mouth during 1½ days urine was collected for analysis. It was found that 8 per cent of the total urinary excretion occurred in the first 24 hours, 50 per cent in the second 24 hours (during which period the maximum blood concentration was attained), and 22 per cent during the third 24 hours. The proportion of acetylated form remained almost constant throughout at about 40 per cent of total.

*Clinical observations.* The majority of the patients to whom Irgamid was given were unsuitable for any real therapeutic trial. A number of them were surgical cases in whom improvement might equally well be attributed to the operation.

TABLE 1  
*Irgamid concentration in blood and cerebrospinal fluid*

CASE NO.	HOURS AFTER ADMINISTRATION	CONCENTRATION IN BLOOD		CONCENTRATION IN C.S.F.		METHOD OF ADMINISTRATION
		Free	Total	Free	Total	
18	4	6.8	7.6	4.8	5.0	2 g intra venously + 1.5 g bymouth
19	4	6.2	2.6	3.0	3.0	
20	4	8.6	8.4	3.0	3.2	
21	3	6.4	8.0	5.8	6.0	
15	12	8.8	9.8	5.0	6.4	Course of 3 g Na irgamld intra muscu larly fol lowed by 1 g 4- hourly oral
	38	10.2	11.0	5.6	7.0	
	50			7.6	9.0	
	62	11.6	12.6	9.8	11.2	
	66	17.2	19.0	13.6	14.4	
		Irgamld discontinued				
	110	10.2	11.0	10.8	12.4	
	134	6.5	7.5	8.0	9.0	
	158	2.4	3.4			
	163			2.8	2.8	

Others had infections which are known to be resistant to sulphonamide therapy. Sensitivity of the organism was tested *in vitro* in five cases (table 3). Three of these infections appeared to be improved by a course of Irgamid but no definite conclusion as to the therapeutic value was reached. The two children with relapse of influenzal meningitis recovered completely. Those with bronchopneumonia recovered but showed a slower response than is usual with sulphapyridine. Unpublished results by other workers suggest that the therapeutic efficiency is not high.

*Toxicity.* The toxicity of the drug appears to be slight and practically no ill effects were noted even on administration of full courses totalling 30 to 50 g. Those patients receiving Irgamid parenterally or having only a single oral dose showed no toxic reactions. Only two patients had any subjective symptoms in relation to the administration of the drug. They both felt "rotten" and were

TABLE 2  
Blood concentration reached in patients receiving courses of *ergamid*

CASE NO	AGE AND SEX	INFECTION	TOTAL DOSEAGE	LENGTH OF COURSE	MAXIMUM BLOOD CONCENTRATION			METHOD OF ADMINISTRATION
					Hours after admin.	Free	Total	
			Grams	Days		mg	mg	
1	15 M	Pneumococcal empyema	26	6	48	7.6	9.0	Repeated oral (small dosage)
2	23 M	Actinomycosis	24	6	22	8.4	9.4	
3	26 F	Strep viridans pelvic abscess	26	6	52	8.0	10.0	
4	49 M	Strep viridans pelvic abscess	15	3	56	13.6	16.6	
5	12 F	B. Coli peritonitis	26	6				
6	38 F	Thrombophlebitis	26	3	44	17.2	20.4	Repeated oral (large dosage)
7	15 M	Pneumonia	35.5	6	48	13.6	18.0	
8	53 M	Septic arthritis $\beta$ haemolytic strep	33	6	52	15.6	20.0	
9	39 M	Empyema anaerobic strep	27	6	52	15.6	20.0	
10	37 F	Bronchopneumonia $\beta$ haemolytic strep	51.5	7	40	11.2	14.6	
11	20 M	Pharyngitis $\beta$ haemolytic strep	15.5	1½	40	13.6	18.4	
12	3 M	Influenzal meningitis	37	11				
13	6 M	Bronchopneumonia	14.5	7				
14	5½ M	Bronchopneumonia	22	10				
15	9 M	T.B. Meningitis	22	3½	86	16.0	16.6	Intramuscular + oral
16	6/12 F	Influenzal meningitis	17.75	8	40	18.7	22.3	
17	52 M	Rheumatoid arthritis	18	3	54	5.7	8.1	Repeated intramuscular

TABLE 3  
Sensitivity of organism to *ergamid* in vitro

ORGANISM	CASE NO	SENSITIVITY TO <i>ERGAMID</i> IN VITRO
Haemolytic strep	10	Inhibited by $10^{-4}$
	11	Inhibited by $10^{-4}$
B. Coli	5	Partially inhibited by $1.5 \times 10^{-4}$
Non-haemolytic strep	3	Not inhibited by $2.0 \times 10^{-4}$
	4	Not inhibited by $2.0 \times 10^{-4}$

relieved when the tablets were stopped. They had slight nausea and one of them vomited twice during the course of 26 g. Both developed very slight cyanosis. The white cell counts were determined on all the patients at intervals through

out treatment. In the majority of cases there was no significant change in the value. Three patients showed a slight rise. In only one case was the course interrupted on account of possible toxic effect. After 15.5 g the white blood cells had fallen from 4,000 on the first day to 3,200. Two days after stopping the course the value was 5,000. This is suggestive of a leucopenic effect but the evidence is inconclusive as a blood count was unfortunately omitted before starting the course. In no case were urinary complications encountered.

Six of the patients had previously been given courses of other sulphonamides. Two had developed a sulphonamide rash and two had marked nausea and vomiting. They all tolerated Irganid well.

#### SUMMARY

1 The concentration of N<sub>1</sub>-dimethylacroyl sulphanilamide (Irganid) in the blood and cerebro-spinal fluid and the excretion in the urine have been studied after various methods of administration.

2 High blood concentrations can be easily reached and maintained and with smaller doses than are required with sulphapyridine or sulphanilamide.

3 The toxic effects in the cases studied were practically negligible—this may be correlated with the relatively small degree of acetylation. The drug is readily absorbed and well tolerated by the intramuscular route.

4 There is reason to believe however that the therapeutic efficiency is not high.

This paper is based on a communication to the Therapeutic Trials Committee of the Medical Research Council. We wish to thank Professor L. J. Witts for his help and advice, the physicians and surgeons of the Radcliffe Infirmary for permission to treat patients under their care, and Dr R. L. Vollum for testing the sensitivity of the infecting organisms.

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# EPINEPHRINE TOLERANCE OF THE HEART ALTERED BY THYROXINE AND THIOURACIL

## (CHEMICAL ASSAY OF EPINEPHRINE IN THE RAT HEART)

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A marked intensification of the effects of epinephrine on the heart muscle through the thyroid hormone is a well known phenomenon. Both in intact animals (Steppuhn, Naumova and Ugarova (1), Wise and Hoff (2), Aumann and Youmans (3)), in animals with the heart denervated (Sawyer and Brown (4)) and in the isolated heart (Sadae (5), Lütolf (6)) the pharmacodynamic action of epinephrine was found to be considerably increased after pretreatment with crude thyroid extracts or thyroxine. Also the production of cardiac hypertrophy and myocardial damage by epinephrine was found to be intensified through thyroid extract (Gross and Greenberg (7)). The functional peculiarities of the thyrotoxic heart (tachycardia, increased stroke volume, anoxic electrocardiogram, tendency toward auricular fibrillation and toward anginal symptoms, degenerative changes) are, accordingly, interpreted by some investigators (Wise and Hoff (2), Raab (8a), etc.) as being essentially due to an abnormal sensitivity to epinephrine and to sympathin, the latter of which is formed in the heart itself.

On the other hand, thyroidectomy was found to markedly diminish the sensitivity of the heart to epinephrine in animals (Schermann (9), Sawyer and Brown (4), Shambough and Cutler (10)) and man (Eppinger and Levine (11)). The beneficial effects of thyroidectomy on the heart, not only in hyperthyroidism but also in angina pectoris and certain types of cardiac failure (Lev and Hamburger (12), Blumgart, Levine and Berlin (13), Claiborne and Hurxthal (14), Clark, Means and Sprague (15), Scherf (16), Singer (17), etc.) are, accordingly, attributed by some investigators (Eppinger and Levine (11), Shambough and Cutler (10), Raab (8a)) to a protection of the heart against the cardiotoxic effects of epinephrine and sympathin. The divergent opinion of Riseman and associates (18) regarding angina pectoris is based on criteria unrelated to angina pectoris (blood pressure, pulse rate).

The fundamental importance of the intrinsic cardiotoxic effects of epinephrine and related substances for the pathogenesis of heart disease (angina pectoris, sudden cardiac death, cardiac failure in "hypertensive heart disease" and uremia) has been emphasized in recent years by the detection of excessive amounts of epinephrine and related catechol compounds in the heart muscle and, temporarily or permanently in the blood, of cardiac patients of the above named types (Raab (8a, b, c, d, e)).

Recognition of this basic factor in cardiac pathology makes it appear desirable

to search for means of protecting the heart against excessive discharges and against the effects of epinephrine and sympathin. X-ray treatment of the hyperactive adrenal glands in angina pectoris (Raab and Soule (8b) Hutton (19) Hadorn (20) Schittenhelm (21) Lippross (22)) and thyroidectomy as mentioned above have already proven successful in this respect. Since the fall of 1943 thiouracil has been administered to angina patients by the author with striking results in most of them (to be published separately)

The following experiments were carried out for the purpose of obtaining further information regarding the quantitative epinephrine tolerance of the heart (lethal myocardial concentration) and its possible alterations through thyroxine and thiouracil.

**METHOD** White male rats (except for 9 gray rats table 1) were used throughout. Epinephrine (Adrenaline Parke Davis & Co 1:1000) was injected under the skin of the hind legs (doses of 2.5  $\gamma$ /gm in one deposit, larger doses in two equal deposits on both legs) Thyroxine (crystalline, Squibb, in alkaline solution) was injected subcutaneously in doses of 1 mgm each on three successive days, followed by injection of epinephrine on the fourth day Thiouracil<sup>1</sup> was administered in the drinking water (0.1%) for an average of 51 days, ranging from 20 to 65 days. All animals which did not die spontaneously were killed by crushing the cervical spine and all hearts were analyzed immediately after death

The concentration of epinephrine in the heart muscle was determined with the adrenaline method of Shaw as modified by the author (Raab (8a)) The total colorimetric readings of this method include beside epinephrine and sympathin (Cannon and Lissák (23)) other related catechol compounds such as adrenalone dihydroxyphenylalanine, leukoadrenochrome, epinine etc. The total readings are represented by the term AC (adsorbable chromogens<sup>2</sup>)

Previous investigations have shown that the adrenal medulla as well as other tissues and blood contain epinephrine-like catechol compounds beside epinephrine proper They are responsible for the low denominator of the specific ratio (d.s.r. see Raab (8f)) as found in blood and most tissues including the adrenal medulla. Ascorbic acid also lowers the d.s.r. but its color intensity is too weak to seriously upset the results. In numerous experimental and clinical observations the method was found to yield results well in agreement with existing knowledge and conceptions regarding adrenosympathetic function and pathology (Raab (8f))

Epinephrine as well as related sympathomimetic catechol compounds if injected are eagerly taken up by the heart muscle and can readily be detected there in concentrations proportionate to the injected doses (Raab (8e))

**RESULTS** *Effect of epinephrine alone* Thirty-six normal adult rats were given subcutaneous injections of epinephrine in mounting doses (table 1)

<sup>1</sup> Pulverized thiouracil was kindly supplied by Lederle Laboratories

<sup>2</sup> Not adreno-cortical compounds as in some earlier publications (e.g. Raab (8g)) when the results were erroneously interpreted as including cortical steroids This error was corrected in another paper (Raab (8f))

Spontaneous death with the manifestations of acute heart failure (dyspnea, pulmonary edema, marked dilatation of the heart) ensued in the following distribution

Dosage $\gamma/\text{gm}$	Mortality %	Minutes between injection and death (av)
2.5	13	12
5.0	73	7½
7.5	83	5

TABLE 1

*Determination of epinephrine tolerance of the hearts of normal rats*

SURVIVED (KILLED 20 MINUTES AFTER S.C. INJECTION OF EPINEPHRINE)				SPONTANEOUS DEATHS AFTER INJECTION OF EPINEPHRINE				
Date	Dose	AC (color units per gm of heart muscle)	d.s.r.†	Date	Dose	Minutes of survival	AC* (color units per gm of heart muscle)	d.s.r.†
	$\gamma/\text{gm.}$				$\gamma/\text{gm}$			
7/25/39	2.5	650	0.80	8/2/39	2.5	12	1,958	1.11
7/25/39	2.5	1,439	2.53	7/15/39	2.5	12	1,928	1.05
7/29/39	2.5	1,568	1.73					
7/31/39	2.5	784	2.50	8/8/39	5.0	7	4,773‡	1.18
8/1/39	2.5	1,059	1.11	8/9/39	5.0	9	2,654‡	0.96
8/3/39	2.5	1,172	1.52	8/9/39	5.0	10	3,380‡	1.10
8/5/39	2.5	920	3.07	8/10/39	5.0	5	3,374‡	1.68
8/6/39	2.5	1,467	1.37	8/10/39	5.0	5	1,932‡	1.32
7/16/39	2.5	1,242‡	1.18	8/15/44	5.0	12	2,018	1.06
7/16/39	2.5	1,694‡	1.55	8/15/44	5.0	7	2,243	1.11
8/7/44	2.5	1,379	1.36	8/18/44	5.0	5	2,270	1.02
8/11/44	2.5	1,161	1.14	8/22/44	5.0	8	3,909	1.00
8/11/44	2.5	1,057	1.18	8/23/44	5.0	10	2,688	1.12
				8/23/44	5.0	6	1,917	1.11
8/7/39	5.0	1,636‡	1.66					
8/8/39	5.0	1,619‡	1.48	5/20/44	7.5	6	4,260	1.13
8/14/44	5.0	1,467	1.29	5/22/44	7.5	5	2,598	1.20
8/14/44	5.0	1,452	1.12	5/22/44	7.5	6	5,104	1.26
				5/23/44	7.5	4	6,264	1.29
5/20/44	7.5	1,868	1.16	5/23/44	7.5	5	5,677	1.33
'Average	3.3	1,408	1.54		3.3	7	3,275	1.17

\* The term "AC" stands for adsorbable chromogens (see text p 331)

† The term "d.s.r." stands for denominator of specific ratio (see text p 331)

‡ Gray rats

Thus, the incidence and rapidity of fatal epinephrine intoxication increased with the dosage

All animals which did not die spontaneously within 20 minutes were killed at this time. None of the "surviving" animals showed an AC concentration higher than 1900 color units per gram of myocardial tissue. On the other hand, all animals which had died spontaneously from epinephrine injection showed

myocardial AC concentrations higher than 1900 color units per gm with an average of 3275 col un./gm. The average d.s.r of the "surviving" animals was higher than that of the spontaneous deaths (1.54 against 1.17).

The total average of the myocardial AC of all epinephrine-injected rats of table 1 (2341 col.un./gm) was 124% higher than the average of 49 untreated controls in this and earlier series. The average d.s.r too was higher than the total control average of 1.00.

While these results suggest that a myocardial AC concentration higher than 1900 col un./gm is not tolerated by the heart and consequently fatal the significance of the time factor is not sufficiently clarified by this group of experiments. Therefore, 13 more rats were killed earlier after the injection of epinephrine. They are included in tables 3 and 5. The average dose of epinephrine

TABLE 2  
Effect of thyroxine (3 mg within 3 days) on the myocardial AC concentration

DATE	CONTROLS			THYROID-TREATED RATS		
	Wt. of heart (% of body wt.)	AC (col. un. per gm. of heart muscle)	d.s.r	Wt. of heart (% of body wt.)	AC (col. un. per gm. of heart muscle)	d.s.r
8/ 4/44	0.31	965	0.95	0.32	1.689	1.73
8/ 4/44	0.30	1.483	1.22	0.33	1.001	0.81
8/ 5/44	0.27	844	0.93	0.36	1.126	1.47
8/ 5/44	0.33	846	0.81			
8/ 7/44	0.32	1.570	1.10	0.36	1.514	1.04
8/ 7/44	0.30	1.122	1.11	0.36	1.167	1.10
8/ 8/44	0.32	1.157	1.03	0.37	1.321	1.02
8/ 8/44	0.26	763	1.00	0.31	792	1.01
8/11/44	0.29	1.704	1.18	0.32	1.170	1.03
8/11/44	0.26	1.062	1.37	0.37	993	1.11
Average	0.30	1.153	1.07	0.34	1.196	1.15
Difference					+4%	

administered to these animals (3.07/gm) and the average time elapsed between injection and killing (7 minutes) were about the same as in those untreated animals which had died spontaneously from epinephrine (table 1). Ten out of these early killed thirteen animals showed myocardial AC concentrations below 1900 col.un./gm and in only three the AC concentration was slightly higher (2073 2319 2403). The average d.s.r of these 13 rats was 1.18.

From these findings the conclusion can be drawn that in the normal rat a myocardial AC concentration of more than 1900 col.un./gm. existing for several minutes will inevitably result in death from acute heart failure.

*Effect of thyroxine alone* Ten plain controls and nine thyroxine treated rats were killed in pairs and analyzed on the same days with the same reagents (table 2). Apart from a slight loss of weight (average 8%) through thyroxine, and the customary thyroxine-induced hypertrophy of the heart no significant

differences were found (Similar results had been obtained before with controls and experimental animals examined several weeks apart (Raab (8g)) In this older series, however, the d.s.r. of the thyroxine animals was much higher than in the controls and the heart hypertrophy was much more marked)

*Effect of epinephrine in thyroxine-treated rats* A dose of 2.5  $\gamma$ /gm of epinephrine was injected into ten control rats and into ten thyroxine-treated animals in quadruple experiments (including also plain controls and thyroxine-treated animals without epinephrine, tables 2 and 3) Seventy per-cent of the thyroxine-treated rats died within 3 to 5 minutes following epinephrine injection, while

TABLE 3

*Effect of thyroxin (3 mg within 3 days) on the epinephrine tolerance of the heart (s.c. injection of 2.5  $\gamma$ /gm of epinephrine)*

DATE	CONTROLS					THYROXIN-TREATED RATS				
	Wt. of heart (% of body wt.)	AC (col. un per gm of heart muscle)	d.s.r.	Mode of death	Minutes after inj	Wt. of heart† (% of body wt.)	AC (col. un per gm of heart muscle)	d.s.r.	Mode of death	Minutes after inj
8/ 4/44	0.33	1,142	1.57	killed	4½	0.39	3,472	1.12	died	4½
8/ 4/44	0.30	2,319*	1.11	killed	4	0.36	2,825	1.30	died	4
8/ 5/44	0.30	1,517	1.33	killed	3	0.38	1,729†	1.55	died	3
8/ 5/44	0.31	1,753	1.15	killed	4½	0.36	1,844†	1.28	died	4½
8/ 7/44	0.31	2,430*	1.08	killed	5	0.38	1,858†	1.19	died	5
8/ 7/44	0.33	1,379	1.36	killed	20	0.32	1,135	1.54	killed	20
8/ 8/44	0.35	1,204		killed	4½	0.42	1,804†	1.21	died	4½
8/ 8/44	0.31	1,096	1.15	killed	3	0.40	933†	1.32	died	3
8/11/44	0.25	1,161	1.14	killed	20	0.39	1,015	1.11	killed	20
8/11/44	0.31	1,057	1.18	killed	20	0.38	1,034	1.30	killed	20
Average	0.31	1,507	1.23		9	0.38	1,765	1.29		9
Difference		+31% (over controls, table 2)					+36% (over thyroxin treated rats, table 2)			

\* No spontaneous death despite AC above the critical level of 1900 col un /gm

† Spontaneous death despite AC below the critical level of 1900 col un /gm

‡ Calculated referring to body weight before administration of thyroxine

none of the epinephrine-injected controls died spontaneously within the same time interval and, in contrast to the thyroxine-treated rats, none of these controls, except one, showed the signs of approaching death (gasping, convulsions, pulmonary edema) before being killed

Only two of the seven thyroxine-treated rats which died spontaneously following epinephrine-injection showed a normally lethal myocardial AC concentration, the other five of these seven animals had a myocardial AC concentration of less than 1900 col un /gm (the lowest being 933 col un /gm) The average d.s.r. was about the same in the epinephrine injected untreated and thyroxine-treated rats.

These results permit the conclusion that the thyroxine-treated animals succumb rapidly to myocardial epinephrine accumulations smaller than those which are lethal in normal rats

From an earlier series of experiments without adequate timing of the controls (Raab (8g)) in which six thyroxine-pretreated animals had succumbed to 2.5  $\gamma$ /gm of epinephrine within 3 to 5 minutes, the impression had been gathered that thyroxine enhances the accumulation of epinephrine in the myocardium. However this seems to be true, if at all only to a slight degree (see table 3), probably attributable to the faster blood circulation and speedier absorption of the injected epinephrine in the thyroxine-treated rats.

*Effect of thiouracil alone* No significant differences were found between nine untreated plain controls and ten thiouracil treated rats, examined in pairs,

TABLE 4

*Effect of thiouracil (0.1% in drinking water for an average of 81 days) on the myocardial AC concentration*

DATE	CONTROLS			THIOURACIL-TREATED RATS		
	Wt. of heart (% of body wt.)	AC (col. un. per gm. heart)	d.s.r	Wt. of heart (% of body wt.)	AC (col. un. per gm. heart)	d.s.r
5/20/44	0 26	1 116	0 90	0 26	1 863	1 17
5/20/44	0 28	1 555	1 10	0 26	1 562	1 27
5/22/44	0 27	911	1 04	0 25	1 660	1 41
5/22/44	0 30	826	1 19	0 27	1 056	1 14
5/23/44	0 29	893	0 96	0 29	1 331	1 66
5/23/44				0 31	1 032	1 43
8/14/44	0 27	1 446	1 05	0 28	1 625	1 54
8/14/44	0 28	1 266	1 19	0 34	844	1 20
8/15/44	0 30	1 802	1 12	0 28	1 176	1 27
8/15/44	0 29	1 322	1 10	0 30	1 077	1 08
Average	0 28	1 237	1 07	0 28	1 328	1 32
Difference					+7%	

(table 4) regarding relative heart weight and myocardial AC concentration, but the average d.s.r. was somewhat higher in the thiouracil animals.

Growth of the thiouracil animals was greatly retarded. Their increase in weight during the total observation period was only 53% against 107% in the controls.

*Effect of epinephrine in thiouracil-treated rats.* A dose of 5.0  $\gamma$ /gm of epinephrine was injected into fourteen control rats and into fourteen thiouracil treated animals in paired experiments (table 5). Forty three per cent of the normal controls succumbed to this relatively large dose within 5 to 12 minutes all of these as usual with a myocardial AC concentration above the critical level of 1900 col.un./gm. Only one survived up to 10 minutes with a slightly higher AC concentration (2073 col.un./gm.)

On the other hand, only seven per cent of the thiouracil-treated animals succumbed to the same dose of epinephrine, although seven animals (50%) showed myocardial AC concentrations which would normally have been lethal (up to 3111 col un /gm ), when they were killed after analogous time intervals following the injection of epinephrine

The average myocardial AC was about the same in the epinephrine-injected controls and thiouracil-treated rats but the d.s.r was somewhat higher in the latter

TABLE 5

*Effect of thiouracil (as in table 4) on the epinephrine tolerance of the heart (a.c. injection of 50  $\gamma$ /gm of epinephrine)*

DATE	CONTROLS					THIOURACIL-TREATED RATS				
	Wt. of heart (% of body wt.)	AC (col un per gm of heart muscle)	d.s.r	Mode of death	Minutes after inj	Wt. of heart (% of body wt.)	AC (col un per gm of heart muscle)	d.s.r	Mode of death	Minutes after inj
8/14/44	0 31	1,467	1 29	killed	20	0 27	1,612	1 56	killed	20
8/14/44	0 30	1,452	1 12	killed	20	0 35	2,857	1 61	died	5
8/15/44	0 34	2,018	1 06	died	12	0 26	3,047†	2 14	killed	12
8/15/44	0 31	2,243	1 11	died	7	0 26	2,241†	1 92	killed	20
8/18/44	0 31	2,270	1 02	died	5	0 32	1,608	1 79	killed	10
8/18/44	0 28	1,044	1 41	killed	10	0 31	2,327†	1 13	killed	5
8/18/44	0 29	1,182	1 11	killed	10	0 30	1,362	1 31	killed	10
8/18/44	0 30	627	0 95	killed	10	0 29	1,009	1 29	killed	10
8/22/44	0 33	3,909	1 00	died	8	0 30	2,298†	1 09	killed	10
8/22/44	0 28	1,849	1 06	killed	10	0 29	2,177†	0 81	killed	10
8/22/44	0 28	2,073*	1 09	killed	10	0 34	2,140†	1 07	killed	10
8/22/44	0 28	1,583		killed	10	0 30	3,111†	1 57	killed	10
8/23/44	0 34	2,688	1 12	died	10	0 29	1,650	1 18	killed	7
8/23/44	0 40	1,917	1 11	died	6	0 30	1,309	1 01	killed	8
Average	0 31	1,880	1 11		10 6	0 30	2,053	1 39		10 5
Difference		+52% (over controls, table 4)					+54% (over thiouracil treated rats, table 4)			

\* No spontaneous death despite AC above critical level of 1900 col un /gm

† The same

From these results the conclusion can be drawn that pretreatment with thiouracil increases the tolerance of the heart muscle to otherwise lethal doses of epinephrine without interfering with its accumulation in the heart

DISCUSSION The above described experiments revealed the following facts

(1) Subcutaneous injection of epinephrine in doses of 2.5 to 7.5  $\gamma$ /gm was followed by rapid death from acute cardiac failure in a percentage of experimental animals and with a speed which increased with the dose

(2) Injection of epinephrine was followed by accumulation of epinephrine in a modified form (lowered d.s.r) in the heart muscle

(3) If the total myocardial AC concentration (= basic concentration of adsorbable chromogens which probably consist essentially of epinephrine-like catechol compounds, plus deposited intact and modified epinephrine) exceeded the critical level of 1900 col un./gm. for several minutes, cardiac death ensued in variably in the normal rats.

(4) Thyroxine-pretreated rats succumbed to considerably lower epinephrine doses and myocardial AC concentrations than the controls.

(5) Thiouracil-treated rats survived considerably higher epinephrine doses and myocardial AC concentrations than the controls

Table 6 gives a collective survey of older results and of those described in the series of this paper. Here too the accumulation of epinephrine in the heart muscle after injection and the contrasting effects of thyroxine and of thiouracil upon the epinephrine tolerance of the heart are conspicuous.

It appears probable that the heart-protecting effect of thiouracil is primarily due to its suppression of thyroxine-formation in the thyroid gland (Astwood (24))

TABLE 6  
*Collective survey of older and recent experiments*

TYPE OF EXPERIMENTS AND DOSEAGE OF EPINEPHRINE	NUMBER OF EXPERIMENTS	AVERAGE MYOCARDIAL AC (COL. UN./GM.)	AVERAGE D.S.R.	MORTALITY FROM EPINEPHRINE
				%
Untreated controls	49	1 042	1 00	0
Epinephrine (2.5-7.5 $\gamma$ /gm.)	55	2 095	1 23	50
Thyroxine + epinephrine (2.5 $\gamma$ /gm.)	16	2 072	1 30	81
Thiouracil + epinephrine (5.0 $\gamma$ /gm.)	14	2 053	1 39	7

Clinical implications of the effects of thyroxine and thiouracil on the epinephrine-tolerance of the heart can be readily deduced if the pathogenic rôle of the most powerful intrinsic cardiotoxic agents of the human body epinephrine and sympathin is duly considered.

The behavior of the d.s.r. indicates in general the presence of abnormally large amounts of intact epinephrine (and sympathin) in the heart muscle after injection of epinephrine and also after treatment with thyroxine (older series) and thiouracil alone. However the individual results are rather irregular in this respect. It seems that part of the injected epinephrine is rapidly transformed within the organism into a colorimetrically similar form but with a d.s.r. lower than that of epinephrine proper possibly into Kisch's (25) Omega which is an anoxia inducing and thus probably cardiotoxic, oxydation product of epinephrine.

#### SUMMARY

Injected epinephrine is accumulated in the heart muscle of the rat in a modified form and is readily detectable there by chemical means.

Cardiac death from injected epinephrine is determined by its concentration in

the heart muscle The lethal myocardial concentration was established for the normal rat

It was found to be distinctly lowered by pretreatment with thyroxine and distinctly elevated by pretreatment with thiouracil These opposite alterations of the tolerance of the heart to epinephrine were also demonstrated by striking differences in the mortality of thyroxine- and thiouracil-pretreated rats, compared with normal animals, following equal doses of epinephrine

The clinico-pathological and therapeutic implications of the cardiotoxic effect of epinephrine on the heart sensitized by the thyroid hormone (angina pectoris, sudden cardiac death, etc), and of the opposite protective effect of thiouracil treatment, are briefly referred to

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# THE PHARMACOLOGY OF N ALLYLNORMORPHINE AS COMPARED WITH MORPHINE<sup>1</sup>

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The preparation of normorphine and norcodeine by von Braun in 1914 made practical the synthesis of a series of derivatives differing from morphine and codeine only in the radical substituted on the nitrogen. A large number of such compounds have been prepared by condensing various alkyl and alkylaryl halides with norcodeine. One of these derivatives, N-allylnorcodeine<sup>2</sup> has very striking pharmacological properties (1). It does not produce the typical depressant effects of morphine or codeine in fact moderate doses are almost without effect. Large doses will cause excitement and tetanic effects. However it is an antagonist to the respiratory depression produced by morphine—the depression being prevented or abolished, depending upon the order of administration of the two drugs. These observations have been confirmed (2).

On the basis of the well known fact that morphine is a stronger narcotic and analgesic agent than codeine the synthesis of N allylnormorphine was undertaken (3) with the hope that this compound might retain the analgesic and narcotic potency of morphine but lack the respiratory action. This appears to have been the first attempt to synthesize a nitrogen-substituted normorphine derivative.

The amount of material available from this synthesis was insufficient for adequate pharmacological study. The animal experiments reported below were performed using five grams of N-allylnormorphine prepared for us by Merck and Company<sup>3</sup>. While this work was in progress a publication appeared by K. Unna of the Merck Institute of Therapeutic Research (4) on the pharmacology of N allylnormorphine.

**METHODS** *Chemistry* The chemical intermediate, normorphine used in the preparation of N allylnormorphine was prepared according to the method of von Braun (5). The normorphine was then refluxed directly with an excess of allyl bromide. The product obtained (8) had a melting point of 89–92°C and gave a test for free phenolic hydroxyl with the iodoxybenzoate reagent (6).

<sup>1</sup> Supported in part by grants from the Therapeutic Research Committee of the Council on Pharmacy and Chemistry of the American Medical Association and the Fluid Research Fund of the Yale University School of Medicine.

<sup>2</sup> This nomenclature is accepted by the American Chemical Society in spite of the fact that it introduces an apparent contradiction. Since noralkaloids by definition have no substituent on the nitrogen there should be no N-substituted noralkaloids. Although the term N-allyl N-desmethylnorcodeine would avoid this error the accepted terminology will be used through this report.

<sup>3</sup> We wish to express our gratitude to Dr. R. T. Major of Merck and Company for his cooperation.

A sample of N-allyl-normorphine prepared at our request by Merck and Company had a melting point of 208–209°C (7). Thus there were two substances designated as N-allylnormorphine which had melting points differing by more than 100°C. Re-investigation of our original product for a free phenolic hydroxyl with the  $\text{FeCl}_3$  test led to the conclusion that our procedure had resulted in allylation on the phenolic oxygen as well as on the nitrogen. Thus, in all probability, the original material prepared by us is N-allyl-O-allyl-normorphine. Conclusive proof of this must wait the synthesis of the compound from O-allyl-normorphine.

Using the allylation procedure of von Braun for the synthesis of N-allyl-norcodeine (DRP 289, 274) we have been able to prepare N-allylnormorphine having the same melting point as that prepared by Weijlard and Erickson.

Solutions of N-allylnormorphine were prepared for injection by dissolving a weighed amount of the anhydrous alkaloid (base) in water with the addition of a slight excess of hydrochloric acid. When complete solution had been achieved the excess acidity was neutralized by the addition of dilute sodium hydroxide to a pH of 5 to 6. Distilled water was then added to make the desired final volume. Morphine was injected as an aqueous solution of Morphine Sulfate, U.S.P.

**Animal Experiments** The respiratory actions of the drugs were studied in unanesthetized rabbits. Respiratory rate was recorded by a magnifying tambour activated by a pneumograph placed on the animal's thorax. A nose mask and low resistance valves (8) were employed to control the direction of air flow so that inspiration was from room air and expiration into a collecting device. In the early experiments the expired air was collected by displacement of water from an inverted graduate. In the later experiments collection was in a calibrated Krogh-type spirometer modified from that recommended by Wright (9). The float of the spirometer was made of  $\frac{1}{8}$  inch sheet plastic (plexiglass) instead of sheet aluminum and the damping vane was located under the float instead of on the counterweight arm. The capacity of the float was increased to about 4,000 cc because of the use intended. Kymograph records were obtained by connecting the counterweight to a writing lever. An electrically operated valve mechanism was incorporated so that the spirometer would empty itself when filled, thus permitting essentially continuous recording of respiratory volumes over a period of several hours. As finally completed the system imposed a resistance to expiration of less than 1 cm. water pressure. The assembled apparatus is sketched in figure 1 and the details of the emptying mechanism are shown in figure 2.

The analgesic properties of the compounds were studied by the method of D'Amour and Smith (10). In this method the pain stimulus is heat from a six volt, fifty-candlepower light-bulb focused by an appropriate reflector and lens onto the tip of a rat's tail. The effect measured is the duration of the fixed intensity stimulus required to produce the characteristic twitch response of the tail. Probably the best evidence for the reliability of this method is indirect. The originators found that assay of various opiates by this method gave results

in good agreement with the accepted clinical value of the drugs. After control determinations of sensitivity the drugs were injected intraperitoneally and further observations made at half hourly intervals.

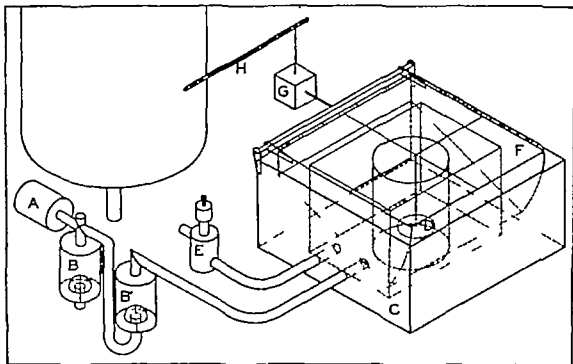


FIG 1 SKETCH OF APPARATUS FOR RECORDING RESPIRATORY VOLUME

A is a mask which is sealed around the animal's nose by a rubber membrane. B and B' are low resistance valves of the type described by Bailey (7) for controlling the direction of air flow. C is the Krogh type spirometer. D is a damping valve operating in an oil bath. E is the automatic emptying valve (see Figure 2); F is the spirometer float made of transparent Plexiglas. G is a counterweight to balance the float; H is a writing lever for kymographic recording.

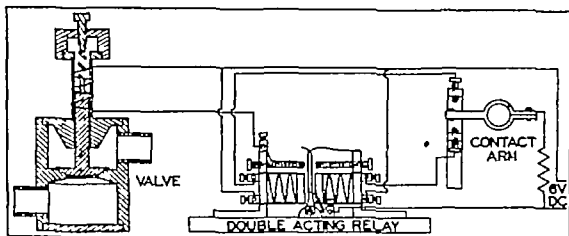


FIG 2 DETAILED SKETCH OF EMPTYING MECHANISM

The contact arm is mounted on the pivot of the spirometer float. The double acting relay is designed to maintain the controlled circuit in the established condition after the controlling circuit is broken i.e. during emptying of the spirometer. The valve consists of a brass body and a soft iron stem which is lifted by the solenoid coil and falls by gravity with a soft spring to insure positive closure.

The effects of morphine and N-allylnormorphine on the intestinal tract were observed on dogs made available to us through the courtesy of Dr C M Gruber and also of Dr J E Thomas. These dogs had Thiry-Vella loops of one or another portion of the small intestine but were otherwise completely normal. The motility of the loop was recorded by inserting a water-filled balloon (15 cm water pressure) and recording the volume changes in this balloon by appropriate means. In these experiments the drugs were injected intravenously into one of the superficial veins of a leg.

Toxicity experiments were performed on mice for economy of material. The drugs were injected either intraperitoneally or subcutaneously in the region of the back as indicated in the results. For each dosage ten previously untreated mice of a stock strain were selected to weigh between 20 and 25 gms.

**RESULTS** *Respiration* The respiratory effects of N-allylnormorphine and morphine were studied on ten unanesthetized rabbits as previously described. At least four experiments were performed on each animal as follows: 1 N-allylnormorphine (5 mgm/kgm), 2 morphine (10 mgm/kgm), 3 morphine followed in 30 minutes by N-allylnormorphine, and 4 N-allylnormorphine followed in 30 minutes by morphine. Consecutive experiments on each animal were at least a week apart and the order of experiments was varied from rabbit to rabbit in order to avoid habituation or conditioning phenomena. The experiment was discontinued when the animal became too restless for reliability. Ordinarily this occurred some five hours after the experiment was originally begun (three to four hours after an injection had been made). Though exact data on respiratory rates and volumes were obtained, the variation from animal to animal and from day to day in a given animal and also the quantitative variation in response to the drugs make it impractical to present any numerical summary of the results. The responses of one typical animal are shown graphically in figure 3.

When N-allylnormorphine was administered to an unmedicated animal there was sometimes, but not always, a brief but intense stimulation of respiration. This was primarily an increase in respiratory rate accompanied by some decrease in tidal volume. The minute volume was occasionally double the control value. Within a few minutes the minute volume decreased to about the lower limit of control values. The decrease was greater on those occasions in which the control value was high than when the control respiration was low. It is difficult to assess the significance of this decrease, but perhaps it indicates tranquilization or some minimal degree of sedation. The slightly low level was maintained for one to two hours following which the respiration returned to the control value. The only other changes observed were late increases in rate and volume of respiration. These were, in all probability, the result of restlessness rather than the action of the drug.

Morphine, in the unmedicated animal, produced its typical depression of respiration. The respiratory rate promptly fell to quite low levels, the tidal volume increased somewhat, and the minute volume fell to about half its control value. These effects persisted about four hours at which time changes due to restlessness occurred. In one case the duration was but two hours.

N allylnormorphine, administered 30 minutes after morphine produced almost the same changes as when given to the unmedicated animal. The above description of the sequence of events following administration of the allyl compound alone was not significantly altered by the existing morphine action. During the periods of decreased respiration the volume approached, but rarely reached, the low level produced by morphine. In most cases the value remained in the low normal range. Thus the respiratory effects of morphine were completely antagonized by N allylnormorphine.

Morphine administered 30 minutes after N-allylnormorphine was practically without effect. Often there was a slight period of increased respiration during and for a minute or two following the injection. This most probably resulted from excitement incident to the manipulation rather than from a drug action. Thus the respiratory effects of morphine were completely prevented by premedication with N allylnormorphine.

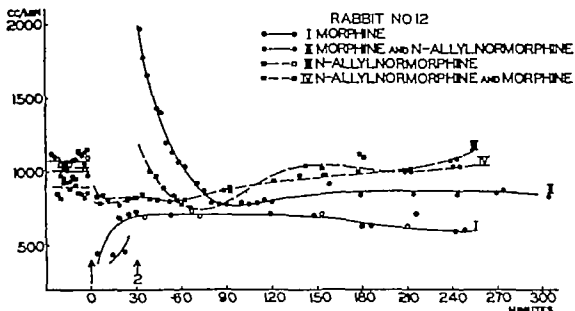


FIG. 3 THE EFFECTS OF INTRAVENOUS ADMINISTRATION OF MORPHINE (10 MG./KG.) AND N ALLYLNORMORPHINE (5 MG./KG.) ON THE VOLUME OF RESPIRATION

Curve I = Morphine injected at 1. Curve II = Morphine injected at 1 followed at 2 by N-allylnormorphine. Curve III = N allylnormorphine injected at 1. Curve IV = N allylnormorphine injected at 1 followed at 2 by morphine.

These results are in agreement with Unna's findings (4) though he studied only changes in respiratory rate.

**Analgesia.** In order to avoid changes due to habituation or tolerance in this portion of the investigation half the rats were tested with morphine on the first experimental day and with N-allylnormorphine on the second experimental day about two weeks later the other half of the animals received the drugs in the reverse order. Control observations were made on each animal shortly before each injection. The results are given in table 1.

Since this is a relatively new method and the interpretation of results is not standardized two approaches to evaluation of the data were used. First, by comparing the means and their standard deviations it seems obvious that both morphine and N-allylnormorphine raised the pain threshold a significant de-

TABLE 1

*Effect of morphine and N-allylnormorphine on response time*

MORPHINE (3.8 MG./KGM. = 10 MICROMOLS/KGM.)			N ALLYLNORMORPHINE (1.55 MG./KGM. = 5 MICROMOLS/KGM.)		
Control (Av of 3 trials)	Maximum	Change	Control (Av of 3 trials)	Maximum	Change
<i>seconds</i>	<i>seconds</i>	<i>seconds</i>	<i>seconds</i>	<i>seconds</i>	<i>seconds</i>
3 4	6 0	+2 6	3 9	4 2	+0 3
4 4	5 2	+0 8	8 0	10 0	+2 0
3 7	5 2	+1 5	4 9	5 8	+0 9
3 7	5 2	+1 5	6 1	7 8	+1 7
4 2	10 0	+5 8	8 0	10 0	+2 0
3 9	5 6	+1 7	6 7	9 2	+2 5
4 3	4 0	-0 3	5 6	8 2	+2 6
3 9	7 8	+3 9	5 5	8 0	+2 5
4 6	5 6	+1 0	7 9	10 0	+2 1
4 1	10 0	+5 9	6 7	6 6	-0 1
4 7	9 2	+4 5	5 9	6 8	+0 9
4 5	10 0	+5 5	5 9	5 6	-0 3
4 7	5 6	+0 9	6 2	6 6	+0 4
4 4	9 4	+5 0	5 3	10 0	+4 7
4 3	8 0	+3 7	7 3	10 0	+2 7
3 9	4 6	+0 7	4 7	5 6	+0 9
4 1	10 0	+5 9	5 3	5 6	+0 3
5 3	10 0	+4 7	7 4	10 0	+2 6
4 7	5 8	+1 1	5 3	7 6	+2 3
3 8	10 0	+6 2	4 9	7 2	+2 3
6 7	10 0	+3 3	4 7	10 0	+5 3
5 1	9 2	+3 9	4 4	5 8	+1 4
6 3	9 4	+3 1	4 9	6 4	+1 5
6 3	8 8	+2 5	4 4	4 8	+0 4
6 2	6 6	+0 4	4 9	6 4	+1 5
4 5	4 6	+0 1	4 1	5 2	+1 1
7 3	8 4	+1 1	5 4	6 8	+1 4
5 0	5 6	+0 6	3 7	4 0	+0 3
6 3	7 8	+1 5	4 1	6 8	+2 7
5 0	6 4	+1 4	5 3	9 8	+4 5
4 5	5 4	+0 9	3 9	4 6	+0 5
8 9	10 0	+1 1	5 9	8 4	+2 5
5 4	6 8	+1 4	4 1	5 0	+0 9
4 5	5 6	+1 1	4 5	5 4	+0 9
7 3	7 4	+0 1	4 3	6 8	+2 5
6 1	9 8	+3 7	4 6	7 4	+2 8
5 0	6 6	+1 6	4 3	5 6	+1 3
5 1	6 2	+1 1	3 6	4 6	+1 0
4 4	5 0	+0 6	4 1	5 6	+1 5
5 9	8 4	+2 5	4 1	6 0	+1 9
5 7	7 4	+1 7	4 3	4 6	+0 3
Av 5 0	7 4	+2 3	5 2	6 9	+1 7
SD $\pm 1 2$	$\pm 2 0$	$\pm 1 9$	$\pm 1 2$	$\pm 1 9$	$\pm 1 3$

gree It also seems clear that the degrees of analgesia produced by the two drugs in the doses used are not significantly different. Second by analysis of the three control observations on each animal (not included in the table) it was found that a response time as much as 17 seconds above the average for that rat would occur by chance only once in about three hundred trials. Thus an increase of 17 seconds or more was considered significant analgesia. The data were re-examined and a tabulation made of the number of animals showing such an increase following the administration of the drug. By the latter criterion it was found that the two drugs produced analgesia in the same number of animals namely 19 of the 41 used or 46 per cent.

Since both analyses of the data led to the same decision it seems reasonable to infer that N-allylnormorphine in a dosage of 1.55 mgm /kgm (5 micromols per kgm) produced about the same rise in pain threshold as morphine in a dose of 3.8 mgm /kgm (10 micromols per kgm). Since the dosage of N allylnormorphine was only one-half that of morphine it seems that the allyl derivative is at least as potent as morphine in this respect.

Using a different type of pain stimulus on mice Unna found that 'N-allylnormorphine is much less effective than morphine in raising the threshold for pain.

*Intestinal tract* The effects of morphine and of N-allylnormorphine on the tonus and motility of the intestine were studied on trained unanesthetized dogs in which Thury Vella loops had been prepared some time previously. Two of these dogs had loops of the ileum one had a loop of the jejunum and the fourth had a loop of the duodenum. Four different experiments were performed on each animal, each drug being injected alone and each followed by the other. The order of experiments was different for each dog. On each experimental day control observations were made long enough to be sure that the gut was stable as to tonus and activity.

Intravenous injection of morphine (1.0 mgm /kgm) produced the expected prompt and prolonged increase in tone accompanied by a decrease in activity. N-allylnormorphine (0.5 mgm /kgm) caused a decrease in tone with a slight increase in activity. There was one exception to this characteristic effect of the allyl compound. The dog having the loop in the jejunum showed an increase in tone but practically no change in activity. However on another day when N-allylnormorphine was injected (to be followed later by morphine) the characteristic effect was observed.

When morphine was followed by N allylnormorphine the tonus of the gut promptly decreased to somewhat below the control level. This was accompanied by a slight degree of hyperactivity. When the morphine was injected after the allyl compound the decreased tonus was eliminated but the hyperactivity remained.

Thus these two drugs appear to be mutually antagonistic with respect to their actions on the intestinal tract. However unlike the actions on the respiration the administration of N-allylnormorphine does not completely prevent the action of subsequently administered morphine.

*Central nervous system in cats* The subcutaneous administration of N allyl normorphine to two cats failed to produce signs of excitement such as are known to result from the administration of morphine. Since only two animals and one dose (25 mgm per animal) were used these results are not conclusive, but they are in agreement with the findings of Unna.

*Toxicity* The mortality of white mice following administration of various doses of N-allylnormorphine is shown in figure 4. From these curves the LD

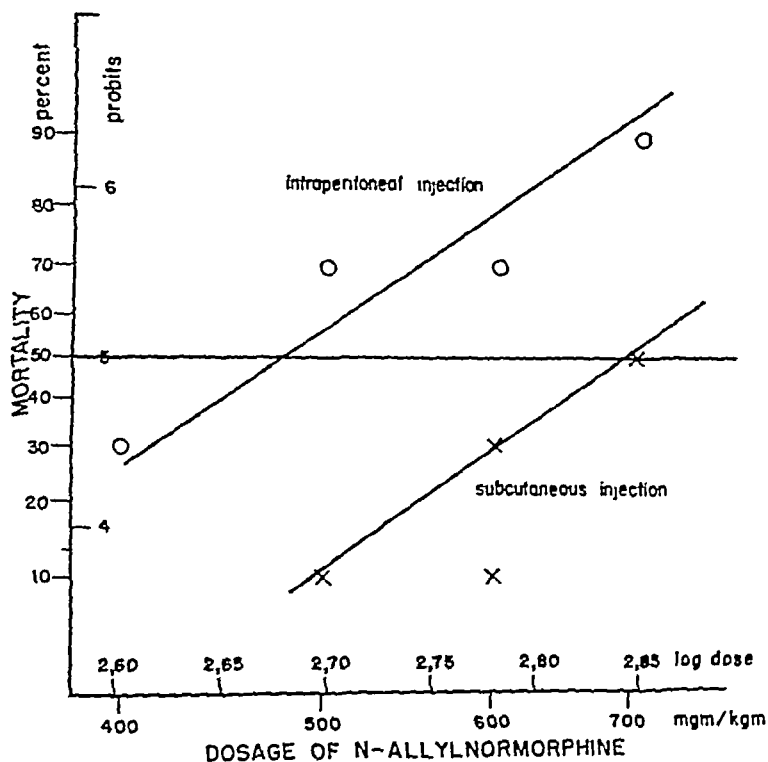


FIG 4 DOSE MORTALITY CURVES FOR N-ALLYLNORMORPHINE HYDROCHLORIDE  
Each point represents ten animals

50 of the compound by intraperitoneal injection is calculated to be  $491.5 \pm 27.8$  mg/kg. The LD 50 by subcutaneous injection is calculated to be  $703.8 \pm 68.4$  mg/kg. The value, 670 mg/kg, reported by Unna for the LD 50 by subcutaneous injection does not differ significantly from our findings.

Lethal doses of N-allylnormorphine do not produce the sedation which is seen following morphine. The Straub reaction was present but not fully typical and death was usually, but not invariably, preceded by convulsions.

\* This figure is standard error. The limits indicated would include two-thirds of all determinations using other colonies of mice under the same conditions.

Since death from acute overdosage of morphine is in some species the result of excessive respiratory depression it seemed desirable to investigate the possibility that N allylnormorphine might decrease the mortality following toxic doses of morphine. For comparative purposes the LD 50 of morphine sulfate was determined on our own colony of mice. The result, 448.4 mg/kg is in substantial agreement with the findings of various other workers. Preliminary

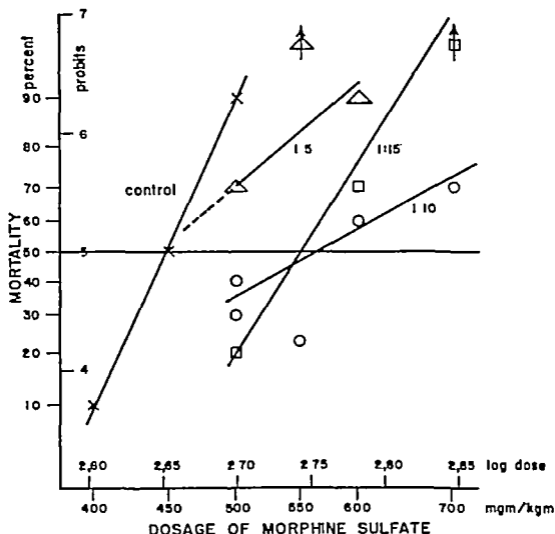


FIG 5 DOSE MORTALITY CURVES FOR MORPHINE SULFATE ALONE AND IN MIXTURES WITH N ALLYLNORMORPHINE

Each point represents ten animals. The curve marked by crosses is a control estimation of the dose mortality curve for morphine sulfate alone. The curves denoted by the other symbols are dose mortality curves for N allylnormorphine-morphine sulfate mixtures in the proportions indicated. Dosage of these mixtures is plotted in terms of morphine sulfate content.

studies using a fixed dose of N allylnormorphine mixed with varying doses of morphine sulfate suggested that the optimum protection is achieved when the N-allylnormorphine and morphine are mixed in a ratio of about 1:12. More careful studies were then made using the two drugs in ratios of 1:5, 1:10, and 1:15. The results of these studies are given in figure 5. Considerable protection is evident.

In addition to the obvious horizontal displacement of the dose-mortality curve

the alteration in slope of the curve is rather striking. The maximum change in slope is produced by the same drug ratio which gives the maximum horizontal displacement. The change in slope may indicate some alteration in the cause of death. This seems reasonable when considered in the light of the known double action of morphine. Although mice are poor test objects for showing the respiratory actions of morphine, they presumably die from a combination of respiratory depression and spinal stimulation. Thus if N-allylnormorphine decreases the respiratory effects it may well "uncover" a wider spread in susceptibility to death from spinal stimulation.

#### SUMMARY

N-allylnormorphine produces a transitory stimulation of respiration followed by a slight depression.

The depression of respiration by morphine is prevented if the allyl compound is administered previously or is abolished if the allyl derivative is given after the morphine.

N-allylnormorphine is at least as potent as morphine in raising the pain threshold of rats.

On the intestinal tract, N-allylnormorphine has actions opposite to those of morphine. However, premedication with the allyl compound does not prevent, but only decreases, the effects of morphine.

The excitement phenomena observed in cats following administration of morphine are not seen when N-allylnormorphine is given in comparable doses.

The LD 50 of N-allylnormorphine for white mice by intraperitoneal injection is 491.5 mgm/kgm, and by subcutaneous injection, 703.8 mgm/kgm. The mortality of mice which have received toxic doses of morphine is decreased by the subsequent administration of N-allylnormorphine.

From these findings it appears that replacement of the N-methyl group of morphine by N-allyl eliminates the respiratory depressant action, does not diminish the analgesic action, alters the action on the intestinal tract, and alters the excitant action in the cat. In most respects the allyl derivative is antagonistic to the parent compound.

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## STUDIES ON ANTIMALARIAL DRUGS

### THE EFFECT OF MALARIA (*PLASMODIUM GALLINACEUM*) AND OF ANEMIA ON THE DISTRIBUTION OF QUININE IN THE TISSUES OF THE FOWL<sup>1</sup>

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In a previous paper we have presented data on the distribution of quinine in the blood and tissues of the normal fowl after oral and intravenous administration (1). The present report deals firstly with the distribution of quinine injected into birds infected with *Plasmodium gallinaceum* and secondly with the influence of experimentally induced anemia on the distribution of the drug in uninfected birds.<sup>3</sup>

**Material and Methods** White leghorns were used throughout. Sixteen birds, mostly cocks, weighing from 1 to 2.7 kg. and three chicks weighing about 200 grams were used in the experiments on infected birds. Ten hens, about 2 kg. in weight, were used in the anemia studies. Eight birds, five adults and three chicks, served as controls.

The birds were infected by blood passage, with the exception of numbers 3105, 2820, and the chicks, which were injected intravenously with sporozoites obtained from the salivary glands of *Aedes aegypti*. Three of the birds (3105, 3116, 3115) were sacrificed when parasites were first demonstrable in blood smears, seven days after infection in the case of 3105 and after one day in both 3116 and 3115. Five birds (3485, 3489, 326, 2932, 3135) were sacrificed during the acute stage of infection, with parasite counts ranging from 65 to 90% parasitized red cells (fig. 1). Eight birds (319, 3484, 2934, 3486, 3491, 3490, 3487, 2820) were recovering from infections, the details of which are given in table 1.

Anemia was produced either by bleeding on successive days or by the intravenous injection of phenylhydrazine, 30 mgm./kg. (2). The degree of anemia was estimated by red blood cell counts and by hematocrit determinations.

The adult birds received 10 mgm./kg. of quinine as the hydrochloride intravenously. The chicks received 20 mgm./kg. intravenously. One hour later the tissues were analysed for quinine by the method of Kelsey and Geiling (3).

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<sup>3</sup> Recently Andrews and Cornatzer (5) reported that in dogs hemorrhagic anemia caused an increased blood quinine level after a single oral dose of quinine.

**Results** The distribution of quinine in the tissues of normal and of infected adult birds is shown graphically in figure 2, while the distribution in the chicks is summarized in table 2. In the figure, each solid bar represents the quinine content in one bird expressed as mgm/kg of moist tissue. The results are

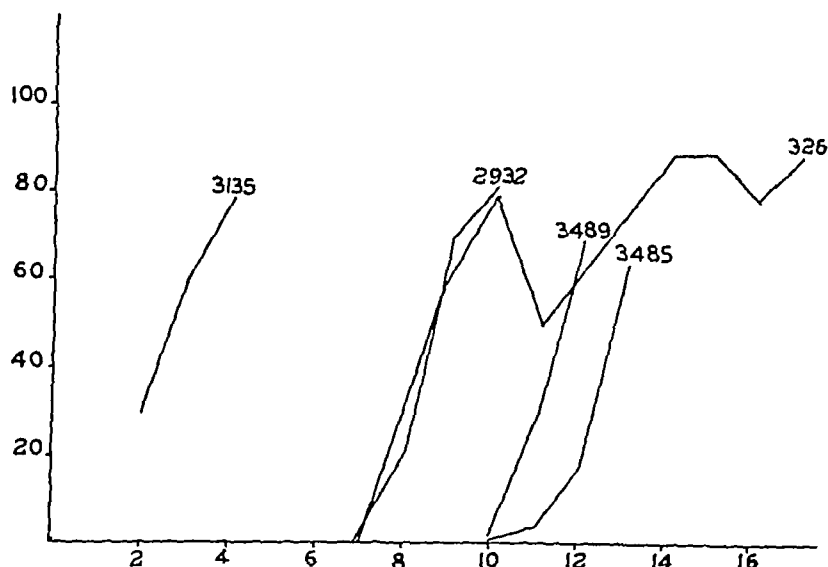


FIG 1 DEGREE OF PARASITEMIA OF BIRDS WITH SEVERE *P. GALLINACEUM* INFECTIONS

The ordinate represents percent of infected red cells, the abscissa, the number of days after the injection of the parasites

TABLE 1

*The severity and duration of infection of the eight birds recovering from P. gallinaceum infections*

NO	DAYS INFECTED	DAY OF MAX. INFECTION	% CELLS INFECTED AT CRISIS	% CELLS INFECTED AT DEATH
319	19	7	10	<1
3484	11	9	45	5
2934	16	13	5	2
3486	16	13	28	<1
3491	14	10	25	<1
3490	20	10	10	<1
3487	20	9	8	<1
2820	39	10	88	<1

arranged so that the first series of bars in each tissue square represents the values found in control birds, the second series, those of birds during early infection, the third, those of birds at the acute stage and the fourth, those of birds recovering from the infection

The quinine concentration observed in normal birds was relatively constant within the same age group but a much lower concentration was observed in the chicks than the grown birds although the former had received twice as large a dose. Apparently chicks metabolize quinine at a much faster rate than do the older birds. *In vitro* experiments were done to see whether a greater activity of quinine oxidase could be demonstrated in the younger birds (4). However, in both adults and chicks only the kidney showed any quinine destroying activity the order of activity being the same in each case.

The tissue quinine concentrations in the infected birds are much more variable than those of the controls and are frequently much higher. This is especially noticeable in the spleen, bone marrow, plasma and red cells and to a lesser extent in the brain, muscle and liver. Except in the plasma and in the red cells, these differences are seen during the acute and recovery periods rather than during the early stage of the disease. The results on the sporozoite in

TABLE 2

Distribution of quinine one hour after the intravenous injection of 20 mgm./kg. into six young chicks. No. 4101, 4105 and 4109 were injected eight days previously with *P. gallinaceum* sporozoites. No. 4102, 4104 and 4106 were normal control birds. The data are given in mgm. of quinine per kg. of tissue.

Bird No.	INFECTED			NORMAL		
	4101	4105	4109	4102	4104	4106
Weight (grams)	305	185	168	300	190	170
% Parasitized cells.	35	47	32			
Spleen	34	76	65	13.5	18.2	13.1
Kidney	9.2	16.6	9.5	9.2	6.7	4.6
Brain	5.8	20.5	5.7	5.3	3.7	3.9
Muscle	2.5	9.5	4.2	2.2	1.2	1.2
Liver	16.4	40	21.5	7.8	5.0	6.5

infected chicks suggest that the presence of exo-erythrocytic stages does not influence the distribution of quinine. These birds received one week previously the intravenous injection of the equivalent of two infected mosquitoes and at the time of the experiment, brain smears showed numerous exo-erythrocytic stages. The liver and spleen contained considerably more quinine in all infected birds than in the normal controls but with the exception of number 4105, the concentration in the brain, muscle and kidney were approximately the same in both. Bird number 4105 appeared very weak after the quinine injection which may account for the fact that the concentrations found in its tissues were much higher than those in the other two infected birds.

The quinine concentration in red cells and plasma, together with the red cell/plasma ratio for ten normal birds and fifteen infected birds is summarized in table 3. In the two birds in which parasites were demonstrable in the blood one day after infection (3116, 3115) the quinine concentrations of the plasma were 9.2 and 15.0 mgm./kg. respectively while the average value for normal

birds is 3.4 mgm /kg. The quinine content of the red cells of these infected birds was slightly higher than that of normal birds. In all birds with severe infections, the quinine concentration of the red cells was well above the average of the controls in contrast with the quinine concentration of the plasma which

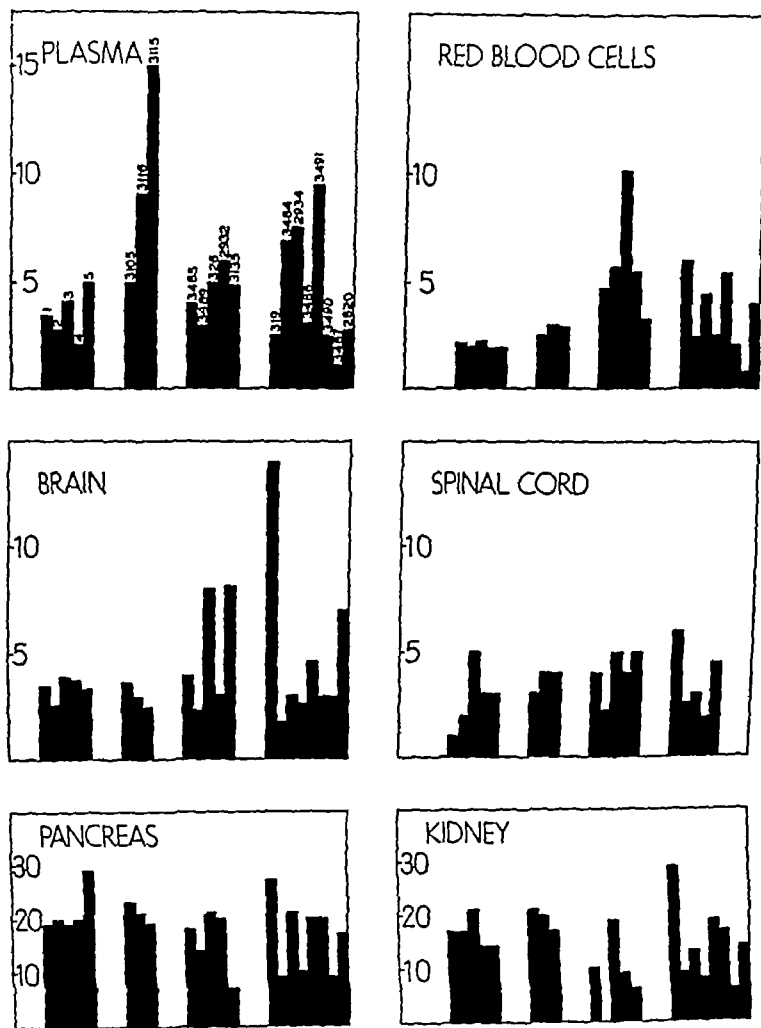


FIG 2A THE DISTRIBUTION OF QUININE IN THE TISSUES OF NORMAL BIRDS AND IN BIRDS INFECTED WITH *P. GALLINACEUM* ONE HOUR AFTER THE INTRAVENOUS INJECTION OF 10 MGm /KG

Each solid bar represents the quinine content of one bird in mgm /kg moist tissue. Birds 1, 2, 3, 4 and 5 were normal controls. Nos 3105, 3116, 3115 had early infections. Nos 3485, 3489, 326, 2932 and 3135 were at the crisis while nos 319, 3484, 2934, 3186, 3491, 3480, 3487 and 2820 were recovering.

fell within the normal range. During the recovery period, however, high values occurred in both plasma and red cells though not always in the same bird.

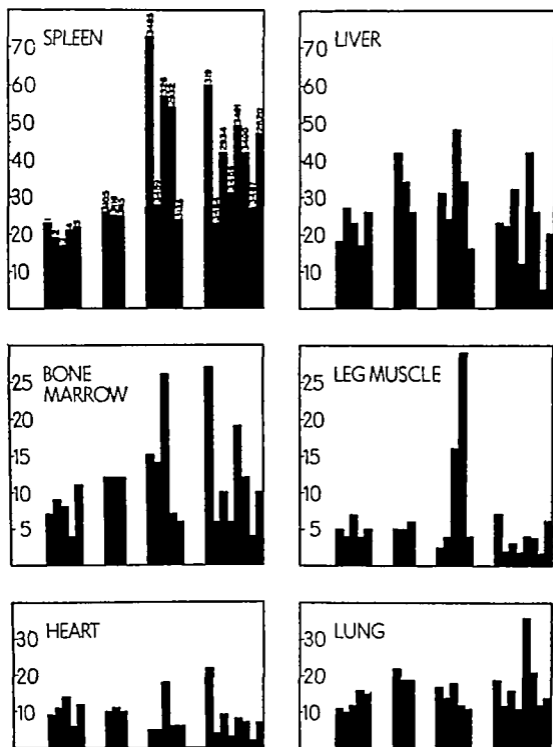


FIG. 2a  
See below fig. 2a

In regard to the ratio of the concentration of quinine in plasma as compared with that of red cells the value in normal birds was found to be relatively con

stant, the average value being 0.6. In contrast, the ratio in the infected birds was more variable and in some birds reached a value greater than one. Since this occurred even in the absence of parasites in the circulating blood (e.g. number 319) the differences cannot be wholly ascribed to a localization of quinine in the infected cells.

Since the infected birds are usually anemic, it was thought advisable to study the effect of anemia *per se* on the distribution of quinine in the cells and plasma.

TABLE 3

Quinine concentrations in the red cells and plasma of normal and infected birds (*P. gallinaceum*) one hour after the intravenous administration of 10 mgm./kg.

	BIRD NO	% CELLS INFECTED	PLASMA mgm./kg	RED CELLS mgm./kg	RED CELL PLASMA RATIO
Normal birds	1		3.4	2.2	0.7
	2		2.7	1.9	0.7
	3		4.1	2.3	0.6
	4		2.1	1.9	0.9
	5		5.0	1.9	0.4
	6		3.8	2.2	0.6
	7		2.9	1.1	0.4
	8		2.5	1.9	0.8
	9		2.4	1.8	0.8
	10		4.9	2.2	0.5
Early infection	3105	1	5.0	2.5	0.5
	3116	8.0	9.2	3.0	0.3
	3115	9.0	15.0	2.9	0.2
High infection	3485	65.0	4.0	4.7	1.2
	3489	67.0	2.9	5.7	2.0
	328	90.0	5.0	10.4	2.1
	2932	82.0	6.0	5.5	0.9
	3135	90.0	4.8	3.3	0.7
Recovered	319	1	2.5	6.1	2.4
	3484	5.0	6.9	2.4	0.4
	2934	2.0	7.6	4.4	0.6
	3486	1	3.1	2.2	0.7
	3491	1	9.5	5.4	0.6
	3490	1	2.4	2.1	0.9
	3487	1	1.2	0.7	0.6

In the first series of experiments, four hens were bled on successive days, 15 to 25 cc of blood being removed by heart puncture. One hour previous to the bleeding, the birds received 10 mgm./kg of quinine intravenously. The red cells and plasma of the withdrawn blood were then analysed for quinine. Red blood cell counts were made prior to the administration of the quinine and hematocrit determinations made at the time of the heart punctures. The data from this experiment are presented in table 3. It can be seen that as the anemia

TABLE 4

The concentration of quinine in the red blood cells and plasma during anemia. Birds were bled on successive days and received 10 mgm./kg of quinine one hour prior to the withdrawal of blood

DAY	WT	HCT	HEMATOCRIT	R.B.C.	PLASMA	RED CELLS	RBC CELL PLASMA RATIO
	kg	g.			mgm./kg	mgm./kg	
1 1	2.1	22	33	2 890 000	2.9	1.4	0.43
2	2.0	24	30	2 160 000	3.4	1.1	0.32
3	2.0	22	23	1 770 000	3.1	1.3	0.42
4	2.0	16	24	1 630 000	4.5	5.5	1.22
5	1.9	21	28	2 000 000	3.9	2.0	0.51
6	1.9	25	26	2 310 000	4.3	2.0	0.46
7	1.9	23	24	1 630 000			
8	1.8	24	24	2 000 000	3.9	1.7	0.44
9	1.8	18	22	1 830 000	4.3	8	0.65
2 1		20	35	2 770 000			
2	2.2	24	26	1 650 000	2.3	2.6	1.13
3	2.1	20	22	1 820 000	2.7	2.7	1.0
4	2.1	23	18	1 480 000	3.5	3.7	1.05
5	2.0	19	21	1 470 000	3.1	5.0	1.0
6	2.0	22	16	1 370 000	6.5	5.7	0.875
7	2.0	24		1 630 000			
3 1	1.9	15	34	2 140 000	2.1	1.7	0.8
2	1.8	22	29	2 250 000	3.2	2.5	.75
3	1.8	25	17	1 770 000	3.3	4.5	1.35
4	1.7	26	17	1 430 000	5.1	5.7	1.11
5	1.65	26	20	1 230 000	5.8	4.7	.81
6	1.6	24		1 630 000			
4 1	1.9	25	40	2 770 000	3.2	2.3	0.72
2	1.9	16	27	2 730 000	3.3	1.9	0.57
3	1.8	22	24	1 970 000	7.6	6.9	0.91
4	1.75	27	20	1 510 000	8.4	4.3	0.51
5	1.7	6.5	18	1 570 000	13.4	7.1	0.53

Control series:—no quinine administered. Whole blood analysed for quinine. Bird No. 1 had none—Birds 2 and 3 showed approximately 0.4 mgm./kg.

TABLE 5

The distribution of quinine in the blood and tissues one hour after the intravenous injection of 10 mgm./kg into birds made anemic by bleeding and by phenylhydrazine compared with the average concentration found in five normal birds

	XENOPHILIC ANEMIA		PHENYLHYDRAZINE ANEMIA		NORMAL
R.B.C. Concentration	1,500,000 24	900,000 16	1,100,000 16	1,800,000 26	
	Quinine concentration in mgm./kg				
Spleen	31	75	65	55	21
Liver	30	59	60	25	22
Plasma	8	10.4	2.4	2.8	3.5
Red blood cells	3.5	3.5	1.6	1.6	2
Bone marrow	11	7	8	4.8	8
Kidney	20	19	18	8.7	17
Pancreas	13	17	11	10	21

progressed, the quinine concentration increased in both red cells and plasma, and in some instances the concentration in the red cells was greater than that in the plasma

In a second experiment, three birds were made anemic with phenylhydrazine, 30 mgm /kg intravenously, while a fourth was made anemic by bleeding. The birds all received 10 mgm /kg of quinine intravenously and one hour later the tissues were analysed for quinine. The results are presented in table 5. A high quinine concentration in the spleen was found in all birds receiving phenylhydrazine and a high concentration in the liver was found in two of the birds. High values in both plasma and red cells was found in only one. The quinine distribution in the birds made anemic by bleeding was essentially that found in normal birds with the exception of the higher red cell and plasma values.

These results on anemic birds suggest that the increase in quinine concentration in certain tissues of infected birds may be related to the concomitant anemia rather than to the presence of parasites. That the phenylhydrazine-treated birds differ from the bled birds in regard to tissue distribution of quinine is not surprising in view of the different type of anemia produced. The former, with its destruction of red cells is probably more comparable to the anemia of malaria.

#### SUMMARY

The distribution of quinine after intravenous administration in the tissues of birds infected with *Plasmodium gallinaceum* and of birds made anemic by bleeding or by injection of phenylhydrazine has been studied.

In infected birds, the quinine concentration in the spleen, plasma, red cells, bone marrow and liver was frequently higher than in uninfected controls. Higher values were also occasionally found in the brain, leg muscle and lung. No significant differences were noted in the spinal cord, pancreas, kidney and heart.

The increased quinine concentration in infected birds is probably not associated with the presence of parasites in red cells or as exo-erythrocytic stages.

Relatively high quinine concentrations were found in both plasma and red cells in most of the anemic birds, and in the liver and spleen of birds made anemic with phenylhydrazine. The relationship between the anemia of the malaria infections and the fate of the injected quinine is discussed.

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# PHARMACOLOGICAL STUDIES ON SULFAQUINOXALINE

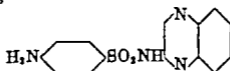
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In an investigation of a series of heterocyclic sulfonamides for activity as suppressive and prophylactic agents against certain species of avian malaria 2-sulfanilamido quinoxaline was found to show promising activity. When compared on the basis of blood concentrations<sup>1</sup> it proved to be as active as sulfadiazine and sulfapyrazine against *P. lophurae* as well as another species of avian malaria and twice as active as these compounds when comparison was made on the basis of weight dosage. It was found that unlike sulfadiazine and sulfapyrazine both of which had to be given three times daily or incorporated in the diet, 2-sulfanilamido quinoxaline would suppress certain avian malaria infections even when administered in single doses at 48 hour intervals. Studies on the pharmacology of 2-sulfanilamido quinoxaline showed that it possessed certain properties not shown by any of the sulfonamides that have been described in the literature. In this communication we are reporting studies on some phases of the pharmacology of 2-sulfanilamido quinoxaline.

The chemistry of 2-sulfanilamido quinoxaline (hereafter called sulfaquinoxaline) is described by Weiglard, Tishler and Erickson (2). The structural formula is the following:



Solubility measurements in distilled water using phosphate buffers to establish the pH showed that sulfaquinoxaline was considerably less soluble than certain of the commonly used sulfonamides.<sup>2</sup> The solubility of sulfaquinoxaline is compared in table 1 with that of sulfadiazine, sulfapyridine, sulfathiazole and sulfamethazine as reported by Rose et al (3). According to these figures the solubility of sulfaquinoxaline is less than that of the other sulfonamides with the exception that at pH 7.9 it is somewhat more soluble than sulfapyridine. The solubility of acetyl sulfaquinoxaline at the physiological range or urinary pH is 10 to 20 times as great as that of the base. The acid ionization constant of sulfaquinoxaline was found by use of the method of Bell and Roblin (4) to have a value in water of  $5.9 \pm 0.3$ .

The persistence of sulfaquinoxaline in the blood of the rat and the dog following single oral doses is shown in figures 1 and 2. In both species an appre-

<sup>1</sup> All determinations of the concentrations of sulfonamides in this report were performed by the method of Bratton and Marshall (1).

<sup>2</sup> We are indebted to Mr. W. A. Bastedo of Merck & Co. Inc. for the determinations of the solubility and the acid ionization constant of sulfaquinoxaline.

able concentration remained in the blood 4 days after oral administration. Following intravenous administration of the sodium salt the behavior was much the same as following oral administration. There was, of course, a high initial peak concentration but this dropped off fairly rapidly to a plateau which was maintained for days. Very little of the drug was acetylated by the rat and the monkey following a single dose as protein free blood filtrates subjected to acid hydrolysis had practically the same concentration of diazotizable material as

TABLE 1

pH	5.2	6.0	6.5	7.0	7.5	7.9
	SOLUBILITY—MG/100 ML.					
Sulfaquinoxaline	0.75	1.5	4.3	11.5	37	75
Sulfadiazine	20	20	25	30	54	100
Sulfapyridine	62	62	62	62	63	68
Sulfathiazole	96	96	104	138	200	312
Sulfamethazine		196	207	234	295	372

The solubility measurements were made at  $37^{\circ}\text{C} \pm 0.2^{\circ}$ , and equilibrium was approached both from supersaturation and from unsaturation, in no case was the difference more than 3% of the value found. Except for the lowest point (which represents the solubility in redistilled water, equilibrated in pyrex), phosphate buffers (0.1 M) were used to roughly establish the pH, and the pH at equilibrium was measured with a glass electrode.

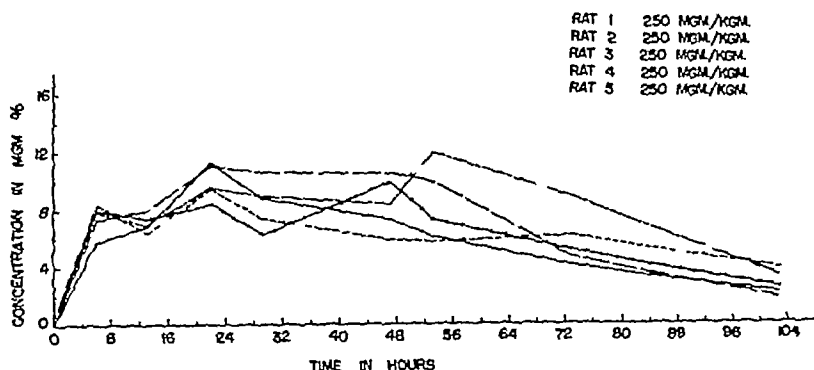


Fig 1 BLOOD LEVELS IN RATS FOLLOWING A SINGLE ORAL DOSE OF 250 MG/100 GM OF SULFAQUINOXALINE

the nonhydrolyzed filtrate, after repeated doses 10 to 20% of the drug was acetylated. The dog showed no acetylation while in the rabbit about 50% of the drug was found to be acetylated.

As the persistence of the drug in the blood stream would lead one to expect the rate of excretion in urine was very slow. However, crystals did appear in the kidney in rats and monkeys receiving sufficient sulfaquinoxaline to maintain a blood concentration of about 10 mgm %. These crystals have been identified by Stevens, Wolf and Pfister (5) as the highly insoluble 3 hydroxy derivative

Scudi and Silber (6) have found that very little unchanged sulfaquinoxaline appeared in the urine, the bulk of the drug was excreted as the 3-hydroxy derivative and a water soluble fraction. It is of interest that sulfaquinoxaline has not been found to produce crystals in the kidneys of the dog and the rabbit.

**ACUTE TOXICITY** Sulfaquinoxaline suspended in 10% gum acacia was administered by stomach tube to 20 gram white mice (Carworth CF1) and to 150 gram white rats (Carworth). The animals were held under observation for seven days following the administration of the drug. As shown in table 2

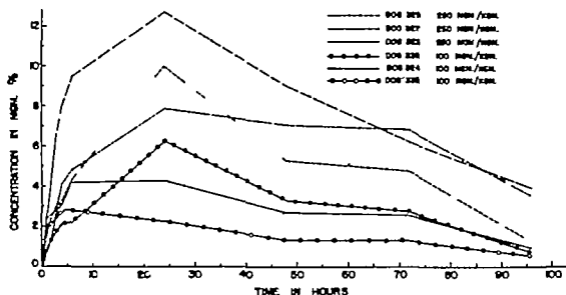


FIG. 2. BLOOD LEVELS IN DOGS FOLLOWING SINGLE ORAL DOSES OF 250 MG./KG. (UPPER 3 CURVES) AND 100 MG./KG. OF SULFAQUINOXALINE

TABLE 2

*Acute oral toxicity of sulfaquinoxaline in mice*

DOSE gm./kgm.	NO. OF MICE	NO. DEAD IN 24 HOURS	NO. DEAD IN 3 DAYS
5	30	0	0
7.5	30	0	1
10.0	40	0	6
12.5	40	0	2
15.0	40	0	7

the acute toxicity of sulfaquinoxaline in the mouse was very low. Only one of 30 animals died following a dose of 7.5 gram/kgm. and but seven of 40 animals died after a dose of 15 gram/kgm., the highest dose that it was practically possible to give.

The acute toxicity in the rat was much greater than in the mouse as four of 10 rats were killed by a dose of 1 gram/kgm. In most of the rats death occurred within the third to the fifth days after dosing. Gross examination of the kidneys of the rats on the higher dose levels showed tiny yellow crystals scattered throughout the cortex and medulla as if the tissue had been sprinkled

with gold dust The deposition of crystals in the kidney was, however, not the immediate cause of death as all animals dying after large doses of sulfaquinoxaline showed hemorrhages into the pleural and the peritoneal cavities of an extent which could be regarded as incompatible with life In addition gross hemorrhages into the tissues of the neck were frequently found and there were ecchymotic areas wherever capillaries might have been injured in the handling of the animals

TABLE 3

*Lowest single oral dose of sulfaquinoxaline producing hypoprothrombinemia in the rat*

DOSE  mgm/kgm	PROTHROMBIN TIMES IN SECONDS			
	Rat no	Control values	24 hrs. after dose	48 hrs. after dose
400	1	15	82	15
	2	12	31	12
	3	14	45	13
	4	13	115	12
	5	15	12	12
	6	14	11	14
	7	15	14	13
	8	16	25	13
	9	16	46	12
	10	17	29	200
200	11	15	16	13
	12	13	13	13
	13	17	12	13
	14	16	12	11
	15	16	11	15
	16	17	12	12
	17	15	12	13
	18	15	14	13
	19	16	13	12
	20	17	15	14
100	21-30 All normal			

It was apparent from the results of the acute toxicity studies that interference with the coagulation mechanism was one of the critical toxic manifestations of sulfaquinoxaline Studies of the platelets revealed no abnormalities Determinations of the prothrombin time, however, showed that single massive doses caused a prompt and marked drop in prothrombin levels (table 3) which in the majority of the rats reached a peak in about 24 hours after a single dose and was back to normal at 48 hours In some of the animals the hypoprothrombinemia developed more slowly and either returned to normal gradually or increased to a point where the animal died of hemorrhage

An experiment was carried out to determine the maximum single dose of sulfaquinoxaline that could be given without effect on the prothrombin level (table 3). It was found that a single dose of 200 mgm /kgm p o did not change the prothrombin time in 10 of 10 rats while 400 mgm /kgm prolonged the prothrombin time in 6 of 10 rats. When vitamin K<sub>1</sub> was given simultaneously with sulfaquinoxaline the development of hypoprothrombinemia was prevented and if given when hypoprothrombinemia was established it would cause prompt recovery.<sup>1</sup>

**CHRONIC TOXICITY** Since a single dose of the drug maintains a reasonably constant blood level for many hours, it was decided to administer it to all animals in single daily doses. All except a few of the animals receiving 100 mgm /kgm or more of the drug were given vitamin K<sub>1</sub> daily to protect against hypoprothrombinemia and thus prevent hemorrhagic death from masking other toxic effects of the drug.

Sulfaquinoxaline is well tolerated by the rabbit. Five rabbits sacrificed after 67 single doses of 500 mgm /kgm over a period of 3 months showed no gross evidence of tissue damage. It is of particular interest that no nephrolithiasis was observed although urinary concentrations as high as 400 mgm. % of total drug occurred.

Three dogs given 100 mgm /kgm daily without protection of vitamin K<sub>1</sub> died of hemorrhage within 7, 8 and 14 days respectively. A second group receiving the same dose of sulfaquinoxaline but with 25 mgm of vitamin K<sub>1</sub> seemed in good health after 3 months of daily dosing. These dogs however had a macrocytic anemia which failed to respond to the parenteral administration of either Reticulogen (Lilly) or a liver extract of high folic acid content. Dogs on lower dose levels had normal blood pictures at the end of 4 months of daily dosing although an occasional dog did show a transient anemia and hypoprothrombinemia during the experiment. As in the rabbit, nephrolithiasis was not observed.

The number of rats on chronic toxicity test, the dose levels, average blood levels and the number of deaths at intervals up to 6 months are shown in table 4. Only the 25 mgm /kgm dose proved non toxic over a period of 6 months. With the exception of rats which died of causes not related to drug toxicity all animals dying during the course of the experiment presumably died because of severe renal damage. On gross examination of the kidneys yellow crystals consisting of 3-hydroxy 2-sulfanilamido quinoxaline were found scattered throughout the cortex and medulla. Very few of the kidneys had crystals in the pelvis stones were rarely found in the ureters or the bladder. On microscopic examination of the kidneys of rats which had received sulfaquinoxaline over periods of several weeks, many of the tubules were found to be severely damaged and in some cases reparative fibrous proliferation was beginning. Apparently as a compensatory mechanism many of the tubules were markedly dilated. The glomeruli in general appeared to be normal. Most autopsies revealed some

A detailed study of the relation of vitamin K like compounds to the sulfaquinoxaline hypoprothrombinemia will be reported by C. W. Mushett and A. O. Seeler.

thyroid enlargement and, in the males, testicular atrophy. Hematological changes were not striking.

Ten *Macacus rhesus* monkeys were given sufficient sulfaquinoxaline to maintain plasma levels of approximately 10 mgm % for one month. Within the first ten days all the monkeys began to show many leucocytes in the urinary sediment but during the entire experiment only a few of the animals had a slight transient microscopic hematuria associated with albuminuria. At time of sacrifice all the monkeys had elevated urea nitrogens and fixed urine specific gravities. In three of the three monkeys tested, the Diodrast clearance was poor. On autopsy the kidneys were found to have crystals scattered throughout the medulla and cortex.

TABLE 4  
Chronic toxicity of sulfaquinoxaline in rats

GROUP NUMBER	NUMBER OF RATS	DOSE	AVERAGE BLOOD LEVEL	NUMBER OF RATS SURVIVING						
				Time in months						
				1/2	1	2	3	4	5	6
		mgm./kgm	mg %							
1	20	400		4	2	0	0	0	0	0
2	20	200	12.2	6	2	1	0	0	0	0
3	20	100	10.3	14	14	8	0	0	0	0
4	20	50	7.0	18	18	18	10	10	10	10
5	20	25	4.5	20	20	20	16	16	16	16
6	15			15	15	14	14	13	12	12
7	10			10	10	10	10	10	10	10

All rats except those in groups 6 and 7 were given a daily dose of sulfaquinoxaline suspended in 10% gum acacia together with 10 mgm of vitamin K<sub>1</sub> in peanut oil. Rats in group 6 were given 10% gum acacia alone and those in group 7 were given 10 mgm of vitamin K<sub>1</sub> alone.

Since nephrolithiasis appeared to be the primary toxic manifestation if the hemorrhagic tendency was controlled by the administration of vitamin K<sub>1</sub>, experiments were performed to learn whether the administration of alkali would be prophylactic. The incidence of renal lithiasis in the rat and the monkey was not reduced even though sufficient sodium bicarbonate was given by mouth to maintain a urinary pH of at least 8.5.

#### SUMMARY

Sulfaquinoxaline is as effective against certain species of avian malaria as sulfadiazine and sulfapyrazine on the basis of blood concentrations and is approximately twice as active when comparison is made on the basis of weight dosage. The drug is remarkable in that it remains in the blood for days whether given by mouth or intravenously.

Sulfaquinoxaline is unique among the sulfonamides reported in the literature in that it is capable of producing a marked hypoprothrombinemia within 24

hours in rats and dogs on a normal diet. In the rat and the dog the effect of the drug on the prothrombin time can be prevented by the simultaneous administration of vitamin K<sub>1</sub>.

In the rat and the monkey kidney damage occurs when the plasma concentration reaches 10 mgm. % or higher. The distal convoluted tubules and the collecting tubules are blocked by crystals of the relatively insoluble 3-hydroxy derivative. The maintenance of a urine pH of 8.5 or above in the rat by the oral administration of sodium bicarbonate does not prevent the precipitation. Nephrolithiasis has not been observed in the dog or the rabbit.

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## THE CHRONIC TOXICITY OF ERGOT

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Ergot is one of the few drugs that has been constantly before the public eye throughout modern times. A heavy infestation of rye, the chief source of ergot, such as has caused epidemics in the past, is rare, and only arises through a coincidence of several circumstances. The chief factor is the atmospheric condition during the flowering period of the rye, at which time the infection of the rye with the ergot ascospores takes place, normally this period lasts for only a week, but it is prolonged by cold and rain. A cold wet season, therefore, not only increases the infestation, but also reduces the yield of the grain. Such a season existed in this country in 1917 when the rye in Wisconsin was infested so heavily that nearly every head had one or more sclerotia and the amount of ergot in the rye in Connecticut ranged from 1 to 5% (1). In addition to the influence of the ergot there appears to be other factors involved in the production of ergotism. Persons who consume large quantities of rye bread often have an inadequate diet, especially a deficiency in vitamin A. Animal experiments, although not conclusive, indicate that a deficiency of this vitamin increases the severity of the convulsive form of ergotism (2). In the present investigation we have demonstrated the influence of protein on a form of ergotism. The most recent extensive human epidemic was reported in Russia following the cold wet summer of 1926 (2). The clinical symptoms were closely correlated with the amount of ergot in the rye. Ergotism appeared wherever there was as much as 1% of ergot present. Rye with 7% of ergot caused fatal poisoning. Freshly harvested rye appeared the most toxic. In an area with a total population of 506,000 2% were affected. In 30% of the cases the condition was acute and lasted for only 3 to 4 days, in other cases the chronic form persisted from 3 to 4 months. The mortality due to ergotism for the whole district was about 0.8%. A mild epidemic occurred in Manchester, England, in 1928 (2). No epidemic has occurred in the United States among humans, however, there have been numerous reports of ergotism in livestock (3, 4, 5).

Ergot came into prominent use by the medical profession in the eighteenth century, although it was apparently used earlier by midwives. It is surprising, therefore, that with this early and great attention to ergot, there has been no thorough investigation of its chronic effects on laboratory animals. Short experiments such as those of Johnson and Palmer (6) on swine and rats show that ergot reduces the food intake and retards the growth of animals. These investigators fed diets containing 0.1, 0.5 and 1% ergot to rats for 12 weeks and 0.5% ergot to swine for 6 weeks. Stockman (7) fed a monkey oatmeal cakes containing 25% ergot for a month, then increased the ergot to 50% and continued

the experiment for 3 months. The animal remained in good health. Two other monkeys were fed for 2 months on cakes made of ergotized rye. They showed no symptoms of ergotism. Pentachew (8) fed an ape a diet containing from 0.5 to 3% ergot for 7 months. No toxicity was observed. Langecker (9) fed ergot to rabbits, mice, rats and guinea pigs. The experiment with rats was the longest and lasted for about 100 days. Seven rats received a gram of ergot each daily except for an occasional day when the normal diet was substituted. Four animals died early but in the survivors no abnormality except cyanosis distal to an experimental trauma on their tail was noticed. Melanby, Surie and Harrison (10) fed from 2 to 5 grams of ergot daily to each of two puppies for 10 weeks. The only harmful effect was that it reduced the food intake; otherwise it was beneficial in being antirachitic. Kaunitz (11) fed crude ergot to 2 cocks. One was fed 180 grams of ergot intermittently for 4 months; the other was fed 250 grams of ergot over a period of a year. Vascular changes occurred in the combs of both birds. In order to extend these incomplete and partially negative results of the feeding of ergot to laboratory animals we have fed small amounts of ergot in different diets for the approximate lifetime of the rat.

**EXPERIMENTAL.** Groups of 20 female rats each from our colony of Osborne-Mendel strain were started at 3 weeks of age on dosages of 1, 2, and 5% of ground crude ergot in our diet A (12), a relatively high protein diet, along with a group of 20 control females. The rats were kept in individual cages in an air-conditioned room for the duration of the experiment.

While these experiments were in progress a second series of animals was started with the same levels of ergot in a low protein diet. There were 18 rats in each group and they were randomized in regard to litter mates. Since it was thought possible that ergot might affect the sexes differently, each group consisted of an equal number of males and females. The ration used in this experiment was composed of cornstarch 72%, casein 6%, corn oil 6%, brewer's yeast 5%, whole liver powder 5%, salt mixture (U.S.P. XII No. 2) 4% and cod liver oil 2%. Since it was learned from the first experiment that some weanling rats refused to eat the food containing 5% ergot, this group was started at a lower dosage (2%) and the level was increased to 5% after 2 weeks.

The ergot used was a mixture of pooled lots of imported crude ergot to give a total of about 40 pounds, which lasted for the duration of the two series of experiments. The ergot was kept in a tightly covered container since it has been shown that crude ergot loses its activity very slowly, as measured either by the U.S.P. cock's comb method or by the colorimetric assay for total alkaloids when so stored. Since ground ergot deteriorates fairly rapidly (2) we ground small lots when needed.

**RESULTS.** *Effect of Ergot on Early Growth.* To have the analyses correspond in all series of experiments a study was made of the effect of ergot on growth rate during the fourth to the fifteenth week inclusive. The rats were 3 weeks of age when placed upon the experimental diet but in order to simplify handling the rats the examination at the end of the first week was advanced or retarded

by several days in some cases, so that individuals could be weighed on the same day of the week. Hence, the time between the first and second weighing varied in length from 4 to 10 days and was omitted from the calculations. Also in the second series of experiments the rats in the group with the 5% ergot were first placed on a diet with 2% ergot and were continued on this diet for 2 weeks before being placed on their final diet. After one week on the diet with 5% ergot, they apparently had become adjusted to the new regime and were eating more or less regularly. By terminating the curve at 16 weeks, the growth rate had only slightly declined as the rat approached maturity.

Data from which table 1 is taken have been studied by the analysis of variance. In the first series of experiments with female rats and the high protein diet, the analysis showed a highly significant value between the levels of ergot ( $p < .001$ ). When this was broken down into a more detailed study, it was found that only

TABLE 1  
Mean growth gain during the 4th to 15th week inclusive

DOSAGE OF ERGOT	SERIES I DIET A				SERIES II LOW PROTEIN DIET			
	No. of animals	Sex	Mean gain in weight	Standard error of mean	No. of animals	Sex	Mean gain in weight	Standard error of mean
%			grams	grams			grams	grams
0	18	F	136.77	$\pm 4.63$	9	M	232.55	$\pm 13.91$
						F	137.88	$\pm 4.67$
1	19	F	133.78	$\pm 2.98$	9	M	173.33	$\pm 15.17$
						F	136.22	$\pm 6.11$
2	20	F	132.00	$\pm 4.80$	9	M	146.22	$\pm 8.03$
						F	116.33	$\pm 9.77$
5	16	F	93.31	$\pm 4.00$	9	M	88.44	$\pm 10.10$
						F	93.11	$\pm 5.57$

the 5% level of ergot was significantly different from the control group. The mean growth values (table 1) for the 1% and 2% levels are slightly below the controls but the differences are not significant ( $p < .05$ ).

In the low protein series not only is there the normal difference in growth rate between the sexes to ergot treatment, but the difference in response between the sexes is significant. In all instances ergot is more toxic to the male rats. Growth rates, except for the female rats on the 1% ergot, are significantly different from the controls. At the 1% level ergot retards the growth of male rats. On this low protein diet a slightly more toxic effect was observed than on the high protein diet, where no effect was seen even at the 2% level of ergot.

*Effect of Ergot on Late Growth.* At 6 months of age the growth rate decreases to a fairly even increment, approximately one-fifth of that during the period between the fourth and sixteenth weeks. Individual growth curves showed a linear trend, therefore, the increase in weight of the rats for the interval between

the 27th and 52nd weeks inclusive was computed independently. A few individuals died during this interval but most of them except for the group on 5% of ergot in the first series of experiments lived much longer. The increase in growth for the individual groups therefore, could be considered of equal reliability.

An analysis of variance for the late growth gain of the rats in the first series showed no significant difference from the controls. Only 9 rats survived on the 5% of ergot and although there is a slight difference in growth rate the difference is not significant with the few survivors. The variance of the late growth decreased with the number of survivors.

A preliminary analysis of the 64 observations on the late growth rate for the low protein series showed that the effect on growth was similar to the first period. The variance showed a significant sex difference in response to ergot. Since the individual variation in the early period still might have affected the late growth rate its effect was adjusted by analysis of covariance. The method of analysis followed was that of Bliss and Marks (13). The correction of the observed values of  $(Y^2)$  for the initial variation ( $W^2$ ) is based upon the regression of  $Y$  upon  $W$  as determined from the experimental error. Thus the regression coefficient ( $b$ ) for relating  $Y$  to  $W$  is determined from the row of values for error as  $b = \frac{(WY) \text{ for error}}{(W^2) \text{ for error}}$ . The  $(Y^2)$  for each row of values is adjusted according to the formula  $(Y^2) = b^2 (W^2) - 2b (WY) + (Y^2)$ . The outstanding result of adjusting the mean squares is the effect on the values representing sex susceptibility. Before adjusting the observed mean squares a significant sex difference was indicated and afterwards the value had crossed the margin between significance and non-significance. Thus it is seen that the increased toxic action of ergot on the male animals during the early growth period has now been overcome. In the plateau period both sexes respond alike to the chronic toxic action of ergot on growth.

*Fractionation Study* Our findings in the above experiments raised a number of questions. The chief question concerned the possible constituent or constituents of ergot responsible for the chronic toxic actions. Ergot is a highly complex substance. Nelson and Calvery (14) list 10 alkaloids which have been isolated from ergot. Crude ergot contains also three pigments, numerous carbohydrates, glycerides, sterols, amines, organic acids and bases (2). A thorough study would require the chronic feeding of the 10 alkaloids and some of the other constituents. Many of these are not available in sufficient quantities. A fractionation study using defatted (by percolation with petroleum ether until fat free) ergot, fatty residue and ergotoxine however has been done. Groups consisting of 8 male and 8 female rats were each placed on diets containing 5% whole ergot, 5% defatted ergot, 5% ergot oil, ergotoxine as ethanesulfonate U.S.P. equivalent to the average amount in 5% ergot (ergotoxine ethanesulfonate to furnish 1 mgm. of alkaloid for each gram of whole ergot) and control. These rats also had a low protein diet but after about 6 months it differed slightly from the low protein diet given above. Because of the scarcity of whole liver

powder, it was eliminated from the diet and the starch and casein increased by 2% and by 3% respectively

The most striking features of an initial inspection of the data for the period between the fourth and sixteenth weeks (table 2) were the small size and the short survival of the rats on the defatted ergot, particularly, this was true of the male group. Since the amount of ergot was first weighed out, then defatted, with the oil given to one group and the defatted portion to the other, there was no difference between the amount of ergot in the food of the rats with 5% whole ergot and that of the rats with the 5% defatted ergot. In general the amount of oil in a batch varied from 30 to 35%. Both the animals in the 5% defatted ergot group and those in the 5% whole ergot group ate much less food than those in the other groups, however, there was no appreciable difference between these

TABLE 2

*Mean growth gain during the 4th to 16th week inclusive for rats on the fractionation study low protein diet*

DRUG	DOSAGE IN TERMS OF WHOLE ERGOT	NO OF ANIMALS	SEX	MEAN GAIN IN WEIGHT	STANDARD ERROR OF MEAN
	%			grams	grams
Ergot	5	8	M	50.38	±17.18
		6	F	73.83	±16.92
Defatted Ergot	5	4	M	9.00	±6.09
		8	F	50.89	13.36
Ergot oil	5	8	M	217.62	±12.80
		8	F	121.75	6.63
Ergotoxine	5	8	M	179.50	±11.20
		8	F	119.62	7.01
Control	0	8	M	235.25	±10.36
		8	F	131.25	±6.49

two groups. In both groups the male rats grew slower than the females. The increased toxicity of ergot when the fatty portion is removed confirms an earlier experiment from this laboratory. Morris (15) found that 1% of defatted ergot retarded the growth of male rats on our standard diet A and that 1% of whole ergot had no effect on growth. Further study of the data by the analysis of variance brought out the fact that there was also a difference in susceptibility between the sexes in the ergotoxine group. In this group the male animals were significantly smaller than the controls ( $p < 0.1$ ). Ergot oil did not affect growth.

Since a large number of the rats from the groups on whole ergot and defatted ergot died or were rapidly losing weight previous to death the growth gains in the late growth period were unreliable. At the end of the year, the groups on ergot oil and ergotoxine were not significantly different from the controls.

Indeed during the late growth period the male rats on ergotoxine had increased their rate of growth sufficiently to make up for the retarding effect of the drug during the first weeks of the experiment.

**PATHOLOGY** Of the 292 rats discussed in this paper 193 were sectioned microscopically 151 of these had been fed ergot and 42 were controls. The routine sections included lung heart liver, spleen pancreas stomach small intestine kidney adrenal testis and ear tumors Uterus ovary bone marrow bone and leg muscles were frequently sectioned From the individual reports a summarization of the important lesions has been made (table 3)

The pathological changes observed fall into three groups—those caused solely by the ergot and not present in the controls those present to some extent in the controls but to a greater extent in the treated animals and those caused by inadequacies in certain of the diets and present equally in control and treated animals In the first group are three lesions—neurofibromas of the ears necrosis and calcification of the lower ends of the renal pyramids and enlargement of the ovaries from marked corpus luteum hyperplasia These lesions were noted in 46 45 and 41 of the 218 treated animals respectively and in certain groups were present in a majority of the animals. In several thousand other rats fed a large variety of substances other than ergot and often showing severe damage from the substance fed these three lesions have been almost nonexistent, ovarian enlargement from corpus luteum hyperplasia has been seen on rare occasions, neurofibromas of the ear have been seen twice and necrosis of the tip of the renal pyramid has never been seen.

In the second group of lesions for which ergot is partly responsible are calcified renal tubular walls and tubular casts, most numerous around the corticomedullary junction and perhaps focal hyperplasia of the squamous epithelium of the proventriculus (forestomach) In the third group resulting from certain nutritional inadequacies, are fatty degeneration of the liver testicular and general visceral atrophy brown pigmentation of the uterus and focal myodegeneration The last two of these together with a certain portion of the testicular atrophy and perhaps to some extent the focal squamous epithelial hyperplasia in the stomach probably represent a vitamin E deficiency with a quite typical histologic picture The fatty degeneration of the liver was noted only in the groups on low protein diets was almost as pronounced in control as in treated rats and is a typical low protein dietary lesion in rats The testicular atrophy was greatest in the groups with evidences of E-deficiency otherwise the rather slight degree present could be accounted for by low dietary protein ergot toxicity and old age

The tumors of the ears have all been neurofibromas of a uniform gross and microscopic appearance They have usually been multiple with individual tumors as large as 1.2 cm. in diameter although the usual diameter was from .4 to .6 mm. and microscopically they have often shown nuclear palisading (figs. 1 and 2) Detailed histological description and similar photographs have already been published (10) and therefore the details will not be repeated here These tumors have not occurred elsewhere in rats fed ergot except on the tails

of two animals and a questionably significant example on the gluteal region of another rat, in each of these three instances neurofibromas were also present

TABLE 3

GROUP	RATS IN GROUP	RATS WITH EAR TUMOR	NECROSIS OF RENAL PAPILLA	ENLARGEMENT OF OVARIES	CALCIFIED RENAL TUBULAR CASTS	DYSTIC EPITHELIAL HYPERPLASIA	PIGMENTATION OF UTERUS	TUBULAR ATROPHY OF TESTES	FATTY DEGENERATION OF LIVER	LOCAL NECROSIS OF LEG MUSCLES
Fractionation—low protein diet plus apparent vitamin E deficiency										
5% plain	16	0	+++	++	+++	+	++	+++	+	+ to ++
5% defatted	16	3	++++	++	++++	+	++	+++	0*	+ to ++
5% ergot oil	16	0	0	+	++	++	++	++	+	+ to ++
5% ergotoxine	16	1	0	++	+	+	++	+++	±	+ to ++
Control	16	0	0	0	+ to ++	±	++	++	±	+ to ++
Second series—low protein diet										
5% plain	18	17	+++	++	+++	0	0	+ to ++	++	0
2% plain	18	8	++	++	++	0	0	++	+	0
1% plain	18	2	++	+	+++	0	0	+ to ++	+ to ++	0
Control	18	0	0	0	+	0	0	+ to ++	+ to ++	0
First series—high protein diet										
5% plain	20	0†	0†	+	+	0	0	Females	0	Not sec
2% plain	20	3	One rat	+	+	0	0	Females	0	0
1% plain	20	0	0	0	±	0	0	Females	0	Not sec
Control	20	0	0	0	0	0	0	Females	0	Not sec
Morris—high protein diet										
1% plain	20	0	0	Males	0	0	Males	+	0	Not sec
1% defatted	20	0	0	Males	0	0	Males	+	0	Not sec
Control	20	0	0	Males	0	0	Males	+	0	Not sec

In above columns ± = very slight, + = slight, ++ = moderate, +++ = marked, ++++ = extreme degree of involvement, sec = sectioned

\* Slight central necrosis of the liver was present in this group

† It is possible that the first few ear tumors were not noticed until such time as one became clearly evident. Only 6 rats of this group were studied microscopically, the smallest number of any group, because many died before the last 6 months of the experiment, when a pathologist became available, therefore some examples of necrosis of the renal papilla could have been missed.

on the ears. There have been no metastases of the tumors. The tumors regressed when the feeding of ergot was stopped and resumed growth when it was

begun again a behavior similar to that of certain other induced tumors (17). It will be noted from table 3 that on a 5% dosage the incidence of ear tumors in the three different groups of rats varied from 30% to 94%. This difference may be due to one of several factors or their combination—length of life differences in diet and the fact that a different lot of ergot was used for the fractionation study than for the other groups. The last variable has demonstrated that the occurrence of ear tumors with the first lot of ergot a composite of several samples was not a peculiar or unique circumstance and that the tumors are reproducible in our rats with different lots of ergot. Of the 46 animals with neurofibromas of the ears 37 belonged to groups of rats consisting of equal numbers of each sex 24 of these were female and 13 male a female preponderance which is significant as shown by a  $p$  value of .03. The earliest tumor was noted after 9 months on experiment in the second series. No neurofibromas occurred in the controls.



FIG. 1. GROSS APPEARANCE OF RAT EAR TUMORS INDUCED BY FEEDING ERGOT.

The tumorigenic potency of the ergot seems to have extended beyond the production of a specific tumor. The various spontaneous tumors of our rats mostly malignant in type and of which the commonest are lymphosarcoma of the lung and embryonal sarcoma of the kidney were about doubled in incidence in the ergot fed rats ( $p = .025$ ). Among the 218 treated rats 50 had some form of spontaneous tumor while 8 of the 74 controls were so affected. In connection with this apparent high incidence of spontaneous tumors it must be remembered that the great majority of these rats were from 18 to 24 months of age at the time of death.

The renal papillary necrosis is a peculiar lesion. The lower one-sixth to one-third of the renal medullary pyramid was uniformly necrotic with a relatively sharp transverse demarcation. The earliest stages noted have been uniform necroses of greater or lesser completeness with a moderate accumulation of leukocytes at the line of demarcation and very few of them in the interior of the necrotic area. In the later stages focal calcification and fibrosis were present.

next to the necrotic zone, and the lower medullary collecting tubules were somewhat dilated, the necrotic portion sloughed and new epithelium covered

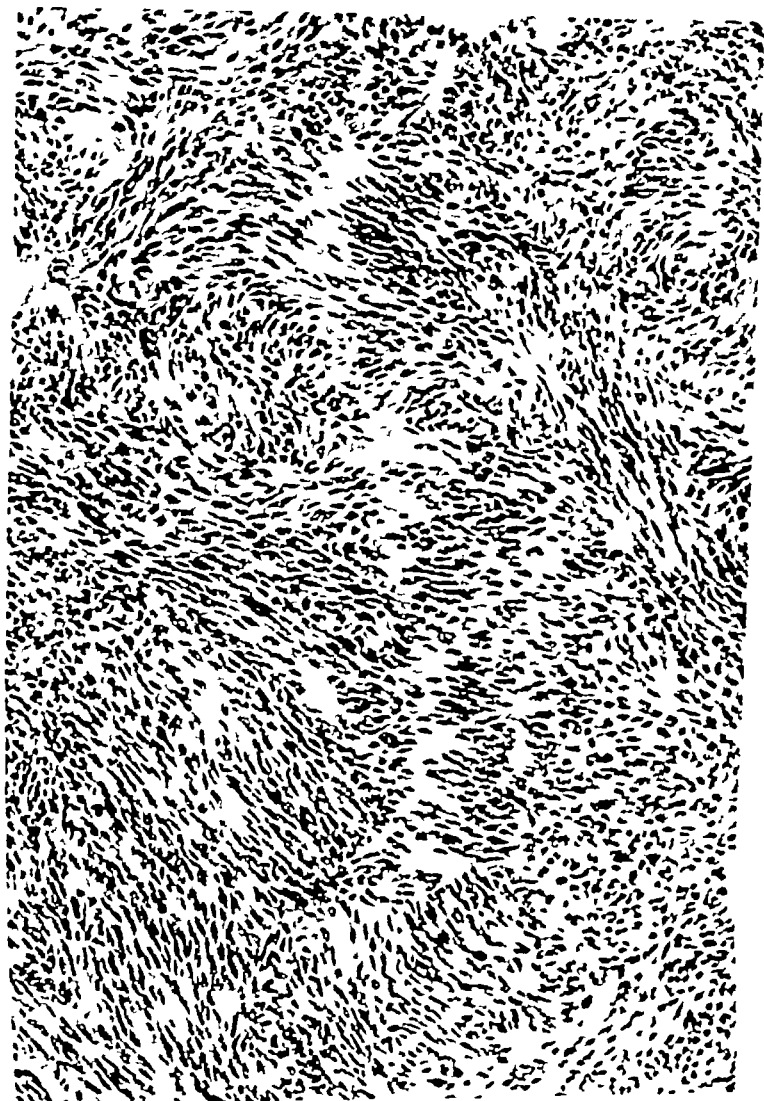


FIG 2 TYPICAL MICROSCOPIC APPEARANCE OF EAR TUMORS, SHOWING NUCLEAR PALISADING CHARACTERISTIC OF NEUROFIBROMA  
X225 Hematoxylin eosin

the ragged stump of the pyramid We have been unable to determine the exact pathogenesis of this lesion It has something of an all-or-none character

In those animals that did not show the lesion the lower end of the pyramid was not particularly abnormal. No vascular changes have been noted in the kidney or elsewhere. Equally damaged kidneys in other series of rats have not shown pyramidal necrosis. The lesion did not occur in the rats receiving ergot oil or ergotoxine so that some other fraction of the crude ergot must be responsible. Gorer (18) noted this lesion as a spontaneous one in certain mice and stated that the origin was obscure. His figure 8 is typical of the condition as seen in our rats. Very recently Dunn (19) also described this condition in mice and stated that it was preceded by amyloidosis. For our rats this does not hold true. Borland and Jackson (20) describe a somewhat similar condition in rats on a strictly fat free diet with no direct statement concerning the pathogenesis; their figure 4 illustrates the condition. In about half the instances which we observed the lesions were relatively recent while in the other half they were relatively old that is recovery appeared possible and the lesion appeared compatible with life. It was usually but not always bilateral. Also it was usually but not always associated with the presence of calcified tubular casts. Both of these lesions were noted as early as 3 months after beginning feeding of ergot.

The enlargement of the ovaries was caused almost entirely by corpus luteum hyperplasia; the follicles and interstitial cells were relatively far less prominent than usual. Whereas the ovaries of the control rats measured around  $5 \times 4 \times 2$  or 3 mm, the ovaries of the ergotized rats measured as much as  $10 \times 8 \times 5$  mm which would mean roughly an eightfold increase in volume, an estimated three- or four fold increase was most common. The corpora lutea were usually in some stage of hyalinization or fibrosis. The hypophysis was not examined. The uterus never showed proliferative changes. The ovarian enlargement was not seen in rats treated for less than  $17\frac{1}{2}$  months; it was present in 41 of the 119 female experimental rats and in one of the 37 female controls.

In the three preceding paragraphs there have been discussed the lesions specifically caused by ergot in this experimental study. Two further lesions, calcified renal tubular casts and testicular atrophy, were in this study partially related to the administration of ergot since they occurred in the treated animals to a greater extent than in the controls. 'Calcified tubular casts' is to some extent a term of convenience since in addition to calcification of the tubular contents the tubular epithelial cells and sometimes the tubular basement membrane were calcified. The majority of the casts were found in the region of the corticomedullary junction with the smaller portion scattered elsewhere in the cortex and medulla. Cortical fibrosis and tubular atrophy were often prominent in the fractionation series in the second series however they were surprisingly slight, although the other renal lesions were not much less severe. The difference is probably related to the greater nutritional deficiency in the fractionation group. Calcified tubular casts in the region of the corticomedullary junction with little other damage to the kidney have been seen as a result of the feeding of other substances such as sulfite and quinacrine.

Squamous epithelial hyperplasia in the forestomach or proventriculus was focal in character and occurred chiefly near the junction with the glandular

portion of the stomach. Microscopically there was hyperkeratosis, thickening of the stratum spinosum with slight to moderate downgrowth, and sometimes subacute inflammatory cellular infiltration in the adjacent submucosa. Occasionally large epithelial pearls were formed, which slightly bulged the serosa. Our impression is that this lesion, while caused to some extent by vitamin E deficiency, is brought out in greater measure if a toxicant or further dietary deficiency is present. We have seen it in well developed form in several dozen rats suffering from vitamin E deficiency and at the same time being fed cadmium, it was less pronounced in a group on an E-deficient diet without cadmium, and it did not develop in rats receiving cadmium and an E-adequate diet. Some of the more marked examples of this change appear to have been regarded as gastric carcinoma in the past (22).

The following four changes had little or no connection with ergot in this study. Brown pigmentation of the uterus typical of that seen in vitamin E deficiency (23) was present in moderate degree in both the control and test animals in the fractionation group, it was noted after 19 months. The only difference between the diet of this group and that of the previous series was the replacement of the 5% powdered whole liver with an increase of 3% in the casein and of 2% in the starch content. Testicular tubular atrophy was slight in degree in the two series on adequate diets, and this slight atrophy is understandable in view of the age of the rats when examined, and also in view of a possible slight toxic effect of ergot on the testis. In the second series the low dietary protein added to the degree of atrophy, and in the fractionation series with an apparent E deficiency in addition to the low protein, the atrophy was of quite high grade. Ergot per se caused but little atrophy, as may be seen by comparison of control and test groups in table 3. The interstitial cells showed no changes of note.

Fatty degeneration of the liver was not present in the groups on a high protein diet, and was slight to moderate in the others, in both control and treated animals. In the defatted ergot group there was slight centrilobular necrosis rather than fatty degeneration. Focal necrosis and replacement fibrosis in the hind leg muscles (other muscles were not studied) was not seen except in the fractionation series, and here it appeared equal in control and treated animals. This finding, together with the increased testicular atrophy and especially the brown pigmentation of the uterus, pointed to a vitamin E deficiency (23).

On the negative side, it is worthy of note that no vascular lesions whatever were seen beyond those spontaneously present in the controls. If vascular changes had anything to do with the ear tumors or the renal papillary necrosis, they were probably functional and not anatomical. The amount of ergotamine in the 5% feeding level was much less than the minimal amount required to produce peripheral gangrene in the rat (24).

**Discussion.** As shown by Johnson and Palmer (6) ergot has a marked effect upon the palatability of a ration. This factor, in all probability, is an important factor in the reduced growth rate observed in the rats on the higher concentrations of ergot. The paired experiments of the above authors, however, showed con-

clusively that ergot retards growth over and above any effect upon food consumption. If the animal does not succumb to chronic ergotism early in the experimental period there is a tendency for the distastefulness to be overcome and the growth rate approaches the normal. This is demonstrated by the refusal of weanling rats to eat a ration containing 5% ergot; on the other hand if they are first placed on a lower concentration for a short time they will eat a ration with the higher concentration.

The fractionation study although limited gives evidence concerning a number of differences between the fractions as will be seen by studying table 3. Ergot oil alone did not cause neurofibromas of the ears and ergotoxine alone was less active in this respect than either crude ergot or defatted ergot. The fact that ergot oil did not cause neurofibromas indicates that the various steroids in ergot, presumably included in the petroleum ether extract were not the responsible agent in their production. A clear-cut difference between crude and defatted ergot on the one hand and ergotoxine and ergot oil on the other was seen in the incidence of necrosis and calcification of the renal papillae. Enlargement of the ovaries, however, was about the same in all four ergot groups.

In this laboratory many dozens of substances such as coal tar colors, organic solvents, inorganic chemical preservatives, etc. have been fed to rats in the same manner as was ergot. Although a number of distinctive lesions such as cirrhosis of the liver, bladder stones, etc. have been produced by chronic feeding, the three lesions described in this paper as specific for ergot have not been seen, with the exception of very rare spontaneous instances after feeding any of these other numerous substances and ergot has been the only substance to produce such a variety of lesions. The latter fact is undoubtedly explained by the greater complexity of ergot.

#### SUMMARY

1. Rats fed powdered crude ergot in an adequate or low protein diet at concentrations of 1, 2 and 5% showed toxic effects.

2. Ergot retarded the growth rate of rats. This effect was more pronounced in male animals during the early growing period. With an adequate diet the 1 and 2% of ergot did not retard the growth of the female animals. On a low protein diet the growth rates except for those of the female rats on the 1% ergot were significantly different from the controls.

3. Histologically typical neurofibromas were produced on the ears of a high percentage of rats fed 5% of ergot. The tumor occurred less frequently on the level of 2% of ergot, rarely on a level of 1% and did not occur in the controls. A low protein diet somewhat favored the production of ear tumors. Tumors other than neurofibroma of the ear occur spontaneously in our older rats and the incidence of these was about doubled in those rats fed ergot.

4. Two other lesions, necrosis and calcification of the lower ends of the renal pyramids and corpus luteum hyperplasia of the ovaries were frequently caused by feeding of the ergot.

5. No cutaneous gangrene and no vascular lesions attributable to ergot were observed.

6 In a fractionation study whole ergot was less toxic than defatted ergot. Ergot oil caused little, or no, toxicity.

7 There is an indication that the alkaloid ergotamine may be slightly toxic in the amount found in 5% of ergot. It slightly retarded the growth rate of rats during the early period.

8 The exact constituent of the crude ergot responsible for the tumor production is not known. The fact cannot be ignored that one rat on the ergotamine did develop a typical neurofibroma of the ear.

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# METHODS FOR THE STUDY OF IRRITATION AND TOXICITY OF SUBSTANCES APPLIED TOPICALLY TO THE SKIN AND MUCOUS MEMBRANES

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The intact skin as an integument for the organism presents a relatively effective barrier to the penetration of many substances. However studies in the field of cutaneous absorption indicate that the skin is permeable in some degree to a great variety of compounds. With increase in medical chemical and technical knowledge, there has been introduced a large number of new compounds for incorporation into preparations for topical application. In addition to ingredients of therapeutic or cosmetic value many preparations for cutaneous application contain surface active (wetting) agents which are capable of altering the physical surface relationships between the absorbing medium (skin) and the substance to be absorbed. For some time this laboratory has been studying the local toxicity to skin and mucous membranes (the functional changes as well as the organic lesions) and the systemic toxicity resulting from penetration through the skin of a large number of organic and a few inorganic compounds. These studies have led to the development of new methods the extension of older ones, and the development of new procedures for the evaluation of results.

In connection with the evaluation of results and their extension to recommendations for large segments of the population the fact is often overlooked that small differences observed experimentally if real become important because of the large numbers involved. For this and other reasons we have used considerable care in planning experiments to avoid bias and as far as possible to prevent those variations due to differences between animals time temperature humidity etc from contributing excessively to the error inherent in quantitative biological experimentation. Bias of judgment in the assignment of numerical values to observed physiological phenomena although entirely unintentional on the part of the observer can greatly influence the evaluation of an experiment. Such bias is minimized if the observer is unaware of the nature of the individual treatments of the lesions and if those treatments are arranged in a balanced design. In addition a properly designed experiment makes possible the use of statistical procedures which may later be desired.

In order to transform qualitative observations of physiological effects to reasonably quantitative objective measurements and in order to obtain data easily subject to arithmetical interpretation we have applied the principle of assigning numerical values to physiological phenomena. An excellent example of this principle which consists essentially of dividing the overall effect into distinct elements the extent or seriousness of which may easily be graded is

given by Friedenwald, Hughes and Herrmann (1) for the objective measurement of injuries to rabbit eyes. We have modified their interpretation slightly and have extended the same principle to the evaluation of other physiological effects. The elements in such a method must be assigned values and weights which properly represent their contribution to the overall picture. Because of the interest manifest in these techniques and procedures during many discussions we are presenting detailed descriptions of those we consider most useful in the evaluation of the relative toxicities of liquids, solids, or solutions topically applied. These include the determination of systemic toxicity following single and multiple dose applications to the skin of different species of animals, primary irritation to the skin and the mucous membranes of the eye and penis of the rabbit, sensitization of the skin of the white male guinea pig, and primary irritation of the skin of man. From the data obtained in these tests, along with that ob-

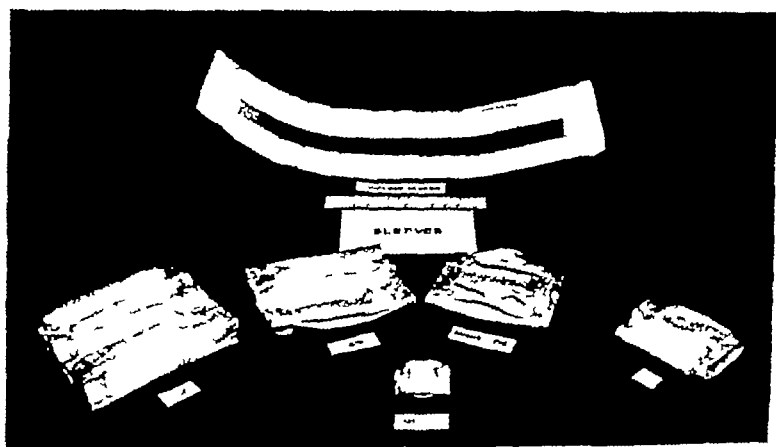


Fig 1 The sleeves are for the animals indicated. The screen is the size used for the rabbit.

tained by histopathologic examination, chemical studies, physiological function tests, and by the administration of the substance by other routes (2, 3), an evaluation of the extent of injury and of the prognosis for repair may be made. After consideration of species differences and an allowance for a margin of safety, the amount, concentration, and frequency of application that may be tolerated by man can be estimated. Some illustrative data are also presented.

**PROCEDURES. Acute Toxicity.** In the acute experiments the agent is held in contact with the skin by means of a rubber sleeve. The sleeve made from rubber dam has been found very satisfactory. It is so constructed that the ends, reinforced with an additional strip of dam (see fig 1), fit snugly around the trunk of the animal. The ends are tucked, permitting the central portion to "balloon" and furnish a reservoir for the dose. The reservoir must have sufficient capacity to contain the dose without pressure. In table 1 are given the dimensions of

sleeves for several species of animals and the approximate body surfaces exposed to the test substance. The sleeves may vary to accommodate smaller or larger subjects of a given species.

Prior to exposure the subjects are prepared by clipping the skin of the trunk free of hair. Approximately half of the animals are further prepared by making epidermal abrasions longitudinally over the area of exposure two centimeters apart. Depilation or shaving causes sufficient disturbance of the stratum corneum so that a 24 hour period is necessary for the recovery of the skin before exposure to the agent. Clipping the hair with small animal clippers is preferred since the intact skin remains undisturbed. In testing some preparations the skin of some animals is moistened with physiological saline prior to exposure and covered.

The sleeve is slipped onto the animal which is then placed in a comfortable but immobilized position in a multiple animal holder (4) as is illustrated in fig. 2. The doses of liquids and solutions are calculated on the basis of body

TABLE 1  
*Dimensions of sleeves for various small laboratory animals*

ANIMAL	MEASUREMENT IN CENTIMETERS		RANGE OF WT OF ANIMALS	AVERAGE AREA OF EXPOSURE	PERCENTAGE OF TOTAL BODY SURFACE
	Diameter (ends)	Overall length			
Mouse	2.5	4.5	20-35	0	8.0
Rat	4.5	9.0	200-350	90	23.4
Guinea pig	6.0	10.5	500-750	90	14.5
Rabbit	7.0	12.5	2,500-3,500	240	10.7
Dog	9.0	12.5	4,000-6,000	500	16.0

The values in this column are for the animals in the middle of the body weight ranges given. The calculations were made by Meeh's formula (G. Luik, *The Science of Nutrition*, p. 122, 3rd Ed. 1928, W. B. Saunders Co., Phila.)

weight and introduced under the sleeve. Preliminary dose ranges of 3.9, 6.0 and 9.4 ml./kgm. are employed. The 3.9 and 9.4 figures represent the antilogarithms of 0.75 and 1.25 times the logarithm of 6.0. Exposures to the agent are for 24 hours. If there is slight leakage from the sleeve which occasionally does occur during the first few hours, it is collected in a glass receptacle and reapplied. A sufficient number of animals at different dosage levels are used so that it is possible to calculate the dose which will be fatal to a given percentage of the animals. This can be determined from the mortality ratios obtained at the various doses (5). The dose which gives a 50-per cent mortality (ID 50) is the one usually calculated. An analysis of the data enables one to determine the slope of the dosage-response curve as well as the probable error of a given determination.

In the testing of materials with unctuous characteristics which adhere readily to the skin, a 20-mesh wire screen is usually employed instead of the rubber sleeve (fig. 1). The screen is padded as illustrated and is raised approximately 2 cm.

from the exposed skin. It is constructed for the various animal species so that the same percentage of body areas are exposed as with the sleeves. The animals are prepared in the same manner as in the exposures with the sleeves. In the case of finely divided amorphous or crystalline solids the material is first evenly distributed on a strip of gauze which is then secured over the area of exposure. The sleeve is fitted over the gauze. In the case of unctuous materials and solids the maximum dose we have used is 4 grams per kilogram.

At the end of the 24-hour period of exposure the sleeves or screens are removed, the volume of unabsorbed dose of liquids and solutions, if any, is measured, and the skin reactions are noted immediately. The subjects are checked for



FIG. 2 Illustrates the exposures in the multiple animal holder. The dog and one rabbit are covered with the sleeve and the other rabbit with the padded screen.

gross symptoms and are transferred to an individual metabolism cage where they are observed for a minimal period of two weeks. Body weight, food consumption and gross symptoms of systemic effects are checked daily. Urine is checked for the presence of protein, reducing substances, blood pigments, etc. Observations for changes in blood morphology are made. The tissues of animals which die several days after exposure and of those sacrificed because of severe poisoning are examined for histopathology.

**SUBACUTE TOXICITY Twenty-Day Experiments** In these experiments relatively large doses are applied daily by intunction to an uncovered area of the clipped skin. The size of the dose employed depends upon the data obtained in the exposures by sleeve. This may vary from 1.0 to 6.0 or more ml./kgm./day.

The area inoculated is approximately 10 per cent of the total surface of the animal. The material is gently rubbed into the skin with a glass rod and this involves manipulation of the hair shafts and mild pressure. The animals are observed for two weeks following the final exposure and the data on mortality ratios are treated statistically as in the acute toxicity experiments. Because of the time factor involved the average survival time of those animals which succumb during the 5-week period furnishes additional data. The repetition of these doses yields valuable information on the action of the agents on the skin as well as progressive systemic effects. In some cases there is a progressive deterioration of the skin so that it becomes a less effective barrier as the doses are repeated. In other cases the agent may produce a mummification or coagulative effect on the skin so that absorption is retarded.

The same observations and tests on the subjects are made as in the acute experiments. The urine and blood are analyzed as above, and microscopic examination of tissues made of the severely poisoned subjects.

*Ninety-Day Experiments* In these experiments dosage levels of 0.5, 1.0, 2.0 and 4.0 ml./kgm. are applied daily for a 90-day period to the clipped intact skin of the animals. The areas inoculated are measured so that approximately ten per cent of the entire body surface is treated. The experiment is similar to the 20-day experiment except that the longer period of treatment with lower dosage levels permits the study of agents which may produce progressive deterioration in the animal. The same observations and tests are made as in the above experiments. In addition tests are made to ascertain whether there is impairment in physiological function of liver or kidney. Special emphasis is placed on blood morphology to ascertain the possible effect of the agent on the hemopoietic organs or on the blood itself. The tissues of all animals on these experiments are examined for histopathologic changes.

The toxicity of more than 200 compounds and preparations intended for topical skin application have been studied in this manner in our laboratory during the past three years. A portion of the toxicity figures for seven compounds studied are given in table 2. It will be noted that there are represented compounds which are very different in chemical nature.

The data in table 2 indicate that compounds differing widely in chemical structure and behavior may be readily absorbed by the skin. There does not appear to be a constant ratio between toxicities by oral and topical administration of compounds. It will be noted also that certain compounds although but slightly toxic as judged by acute exposure (skin) may upon repeated exposure (20- or 90-day) be severely damaging. Compounds which behave in this manner often do not cause gross skin changes at the first exposure but produce severe skin changes upon repeated exposures. The skin injuries range from mild dermatitis to complete necrosis and desquamation. The regenerated skin as a rule is more resistant to the future action of a given compound.

The rate of penetration of the compound into the skin and the irritant effects noted following both the single and repeated applications comprise a portion of the information obtained in these experiments.

**PRIMARY IRRITATION** *Animal Skin* In addition to the observations on primary irritation described above, the following patch test technic is used to measure this quality more precisely. The intact and abraded skin of the albino rabbit is used. The hair is clipped from the back and flanks of the animal. Four areas of the back, indicated in figure 3, placed approximately ten centimeters apart, are designated for the position of the patches. Areas 2 and 3 are abraded by making four epidermal incisions (two perpendicular to two others in the area of the patch). The patches consist of two layers of light gauze cut in squares (2.5 cm on the side). These are secured to the area by thin bands of adhesive tape. The material to be tested is introduced under the patch, 0.5 ml in the case of liquids or 0.5 grams in the case of solids. The entire trunk of the rabbit is wrapped in rubberized cloth (see fig. 3). This helps to hold the patches

TABLE 2  
*Toxicity of some organic compounds for the rabbit*

COMPOUND	LD <sub>50</sub>		
	Oral (acute)	Skin (acute)	Skin 90-day inunctions
	ml /kgm	ml /kgm	ml /kgm
1 Diethylene glycol monobutyl ether acetate	2.6	5.75	3
2 2-ethyl hexanediol 1,3	2.6	10.0	2
3 2-ethyl butanol	1.2	2.0	
4 Dimethyl phthalate	4.4	10	4
5 Dimethyl gamma pyronebutyl-ester	5.3	10	2
6 Isobornyl thiocyanacetate	0.63	10±	1.0 ml /kgm causes death in all subjects from 5 doses
7 Beta butoxy beta thiocyanodi ethyl ether	0.035	0.15-0.25	0.10 ml /kgm causes death in all subjects from 8 doses

in position and retards evaporation of volatile substances during the 24 hours of exposure. Four compounds are commonly tested per series of six subjects. A given compound is applied so that there are three applications to intact skin and three to abraded skin. The sites are assigned as illustrated in figure 4.

The animals are immobilized in the special holder during the 24 hour patch exposure. Upon removal of the patches the resulting reactions are evaluated on the basis of a scale of weighted scores (table 3). Readings are made also after 72 hours and the final score represents an average of the 24- and 72 hour readings.

The table of scores (table 3) gives no values for agents which are severe vesicants or severe escharotics. When such agents are encountered dilute solutions in a bland nonirritating solvent are tested. Sufficient dilution is made so that the responses elicited may be graded by the above scale.

As a rule substances which produce a score of two or less at 24 hours become negative i.e. are completely healed before the 72 hour reading. In table 4 the scores are given for six undiluted organic compounds and one 2% solution.

The averages of the 24- and 72 hour scores for the intact and for the abraded skin, as well as a combined average are shown in the table. The combined average is referred to in this laboratory as the primary irritation index and is useful for placing compounds in general groups with reference to their irritant properties. It should however be kept in mind that without the individual

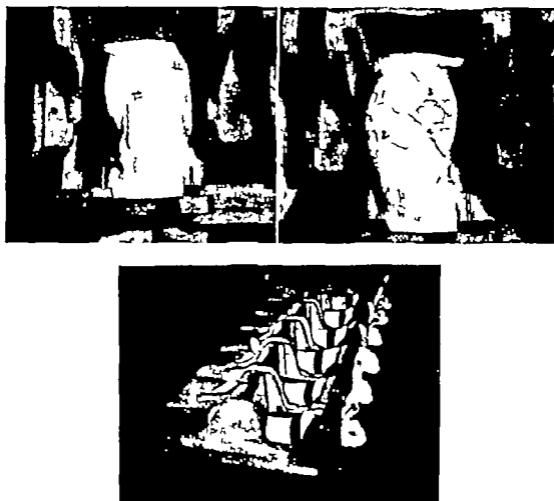
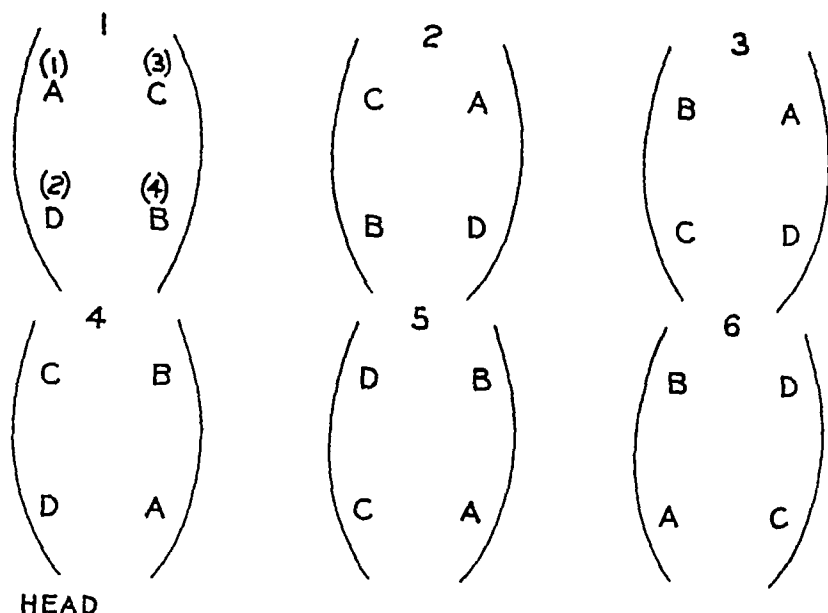


FIG. 3. The epidermal abrasions made in Areas 2 and 3 are shown in 1 (Upper left) and the relative position of the patches is shown in 2 (Upper right). A group of six rabbits covered by the rubberized cloth and in the process of exposure is illustrated in 3 (Bottom)

data the primary irritation index is not in some instances completely informative.

Compounds producing combined averages (primary irritation indexes) of 2 or less are only mildly irritating whereas those with indices from 2-5 are moderate irritants and those above 6 are considered severe irritants.

*Animal Mucous Membrane.* Irritation of mucous membranes is measured on the rabbit's eye and penis. In the case of the penile mucosa the preparation is applied so that thorough wetting is attained. Approximately 0.2 ml of the



## HEAD

FIG 4 Four preparations, A, B, C and D are assigned to the four areas (1), (2) (3) and (4) as illustrated in a series of six rabbits. Areas (1) and (4) are intact skin. Areas (2) and (3) have four epidermal abrasions.

TABLE 3  
*Evaluation of skin reactions*

<b>A Erythema and Eschar Formation</b>	
Very slight erythema (barely perceptible)	1
Well defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4
Total possible erythema score	4
<b>B Edema Formation</b>	
Very slight edema (barely perceptible)	1
Slight edema (edges of area well defined by definite raising)	2
Moderate edema (area raised approximately 1 mm)	3
Severe edema (raised more than 1 mm and extending beyond area of exposure)	4
Total possible edema score	4
Total possible score for primary irritation	8

preparation is required. The erythema and edema are evaluated on a basis of weighted scores which total up to 4 for the most severe injuries, that is, the varying degrees of erythema and edema may each have a possible score of 2.

If a preparation is found sufficiently irritating to cause necrosis and sloughing of the mucosa the agent is reapplied in sufficient dilution so that the resulting injuries total a score of 4 or less. Reactions are usually read at 1-2 and 24 hours after application. Illustrative data are given in table 5.

TABLE 4  
Scores for primary irritation

COMPOUND	RABBIT SKIN				COMBINED AVERAGE
	Intact		Abraded		
	24 hrs.	72 hrs.	24 hrs.	72 hrs.	
Diethylene glycol mono-ethyl ether	0 2 0	0 0 0	0 0 4	0 0 0	0.5
Average	0.3		0.7		
2 methyl 2,4 pentanediol	4 3 0	2 0 1	4 4 2	3 1 1	2.1
Average	1.7		2.5		
Dioctyl sodium sulfosuccinate (2% aqueous solution)	4 3 4	7 2 2	4 1 3	1 0 1	2.7
Average	3.7		1.7		
2 ethyl butyl glyceryl ether	4 3 6	6 0 2	4 5 6	4 4 4	4.0
Average	3.5		4.5		
4 phenyl-3-oxapentanol 1	4 3 5	4 1 6	4 8 8	0 8 8	5.0
Average	3.7		7.0		
3,3,5 trimethyl cyclohexyl dipropylene glycol	6 4 3	6 8 3	5 0 6	7 8 8	5.8
Average	5.0		6.7		
3,6 dioxadodecanol 1	7 8 8	8 8 8	8 8 0	8 8 6	7.6
Average	8.0		7.3		

In the measure of injury to the eye a modification of the scoring system of Friedenwald, Hughes and Herrmann (1) is used. Injuries to the cornea, conjunctival and palpebral mucosae and the iris are scored separately. In testing the materials (liquids, solutions and ointments) 0.1 ml. is instilled in the con-

junctional sac Readings are usually made at 1, 24, and 48 hours after instillation of the agent into the eye and are evaluated according to table 6 Readings are also made after 96 hours if residual injury is present Illustrative data are given in table 7

**Human Skin** When a large series of compounds is involved it is most expedient to use experimental animals in making screening tests for primary irritation After the majority of compounds have been discarded in the screening tests, the few remaining must be tested on man for final evaluation Moreover, a correlation of animal with human data thus made possible is a measure of reliability or applicability of the animal tests

The use of the patch test in the evaluation of primary irritation and of contact dermatitis has been reviewed by Schwartz and Peck (6) We have found similar patch tests to be valuable in the comparative assay of the irritation of various ointments and solvents The general procedure is as follows Two to six

TABLE 5  
*Irritation of the penile mucosa in rabbits*

SUBSTANCE	CONCENTRATION	AVERAGE READING	
		1-2 hours	24-hours
Propylene glycol	15%	0 0	0 0
Ethylene glycol	15%	0 0	0 0
1-(p-arsenosophenyl) urea	2% in propyl ene glycol	0 0	0 5
Alkyl dimethyl benzyl ammonium chlo ride	0 2%	1 0	
Alkyl dimethyl benzyl ammonium chlo ride	2 0%	2 5	4 0
Dioctyl sodium sulfosuccinate	2 0%	2 0	1 0
Dioctyl sodium sulfosuccinate	10 0%	2 0	2 0

substances are chosen for testing with one of these serving as an arbitrary standard of reference Discs of surgical gauze 1 cm in diameter are treated with 0.1 ml of the respective ointments or liquids, then placed at random on the interior surface of either upper arm and covered with glassine paper and adhesive patches Three samples can be placed on each arm with ease After a suitable time interval, usually 24 hours, the patches are removed and the sites cleaned with ether or acetone After allowing 15-20 minutes for any irritation due to the cleansing to subside, readings are made according to the scale employed in the rabbit patch tests

**SENSITIZATION** That eczematous sensitization or contact dermatitis to simple compounds can occur in human beings has been adequately shown (7) The human skin is easily sensitized to such simple compounds as 2,4-dinitrochlorobenzene and nitrosodimethylaniline and less easily to certain coal tar colors (7) The question of whether new compounds have sensitizing properties may become very important especially when the preparation is to be used on the

face or when specialized or skilled procedures may be interfered with because of slight discomfort such as itching resulting from an acquired sensitivity.

The ideal test subject for the determination of sensitization to a compound is man but the use of such subject is usually neither wise nor expedient. The

TABLE 6  
*Scale of weighted scores for grading the severity of ocular lesions*

<b>I Cornea</b>	
A Opacity—Degree of Density (area which is most dense is taken for reading)	
Scattered or diffuse area—details of iris clearly visible	1
Easily discernible translucent areas—details of iris slightly obscured	2
Opalescent areas—no details of iris visible—size of pupil barely discernible	3
Opaque iris invisible	4
B Area of Cornea Involved	
One quarter (or less) but not zero	1
Greater than one quarter—less than one half	2
Greater than one half—less than three quarters	3
Greater than three quarters up to whole area	4
Score equals $A \times B \times 5$ Total maximum = 80	
<b>II Iris</b>	
A Values	
Folds above normal—congestion—swelling—circumcorneal injection (any one or all of these or combination of any thereof)—iris still reacting to light (sluggish reaction is positive)	1
No reaction to light—hemorrhage—gross destruction (any one or all of these)	2
Score equals $A \times 5$ Total possible maximum = 10	
<b>III Conjunctivae</b>	
A Redness (refers to palpebral conjunctivae only)	
Vessels definitely injected above normal	1
More diffuse—deeper crimson red—individual vessels not easily discernible	2
Diffuse beefy red	3
B Chemosis	
Any swelling above normal (includes nictitating membrane)	1
Obvious swelling with partial eversion of the lids	2
Swelling with lids about half closed	3
Swelling with lids about half closed to completely closed	4
C Discharge	
Any amount different from normal (does not include small amount observed in inner canthus of normal animals)	1
Discharge with moistening of the lids and hairs just adjacent to the lids	2
Discharge with moistening of the lids and considerable area around the eye	3
Score $(A + B + C) \times 2$ Total maximum = 20	

The maximum total score is the sum of all scores obtained for the cornea, iris and conjunctivae.

White male guinea pig has been shown by Landsteiner and Jacobs (8) to be a reasonably satisfactory test animal. The procedure used in this laboratory is similar to that used by these investigators and is as follows:

White male guinea pigs weighing 325 to 450 grams subsisting on commercial

rabbit pellet ration supplemented with kale are identified and the hair removed from the back by close clipping. A 0.1% solution or suspension in water of the material to be tested is injected intracutaneously using a 26 gauge needle. The injections are made every other day or three times weekly until a total of ten have been made. The 10 sensitizing injections are made at random in an area about three to four centimeters square just below the midline of the back. The test injection is made on the flank slightly below the sensitizing area. The first injection consists of 0.05 ml while the remaining injections consist of 0.1 ml. Two weeks after the tenth injection a test injection is made using 0.05 ml of a freshly prepared solution or suspension as before. Twenty-four hours later a reading of the diameter, height, and color of reaction is made and compared with similar readings taken after the first injection. If the values for the test readings are appreciably higher than those for the initial readings the substances can be

TABLE 7  
*Eye irritation in rabbits*

SUBSTANCE	CONCENTRATION	AVERAGE EYE INJURY	
		1 hour	24 hours
Diethyl phthalate	Undiluted	3.2	1.5
Dimethyl phthalate	Undiluted	3.3	2.2
Triacetin	Undiluted	2.1	1.5
Propylene glycol	Undiluted	4.8	1.0
Ethylene glycol	Undiluted	6.0	3.2
Ethylene glycol diethyl ether	Undiluted	11.0	13.2
Diethyl sodium sulfosuccinate	0.5%	4.0	2.0
Diethyl sodium sulfosuccinate	2.0%	9.0	2.0
Diethyl sodium sulfosuccinate	10.0%	26.0	24.0
Propylene glycol	15%	0	0
Ethylene glycol	15%	0	0
1-(p-arsenosphenyl) urea	2% in propylene glycol	4.0	3.0

said to have produced sensitization. The degree of sensitization is proportional in part to the increase in those readings.

When dealing with powerful sensitizing agents simple applications or paintings of 0.05 ml of a 1 or 2% solution in some organic solvent such as ethyl alcohol, n-hexyl alcohol, or acetone is sufficient to produce sensitization in guinea pigs. A solution of 0.1% of the material in acetone is usually satisfactory for retest. This procedure is not suitable, however, to produce a response with substances having only moderate or weak sensitizing properties.

**DISCUSSION AND SUMMARY** Some techniques are described for the study of the acute and subacute local and systemic toxicities of substances applied topically to the skin and mucous membranes. The technique for the determination of the local and systemic effects of large single dose applications may at first seem to have little practical application. However, it should be kept in mind

that industrially many organic liquids come in contact with the skin accidentally or otherwise and cause systemic poisoning and even death. Therapeutically it may at times be advisable to make large-dose applications to the skin so that if the relative rate of absorption and systemic effects of a preparation are known, the clinician can be advised of the necessary precautions. The use of special holders, sleeves and screens permits large doses of an agent to remain in contact with the skin for appreciable periods of time and prevents the animal from ingesting any portion of the dose during the exposure. The subacute experiments yield considerable information particularly with reference to irritation and injury to the skin following repeated application as well as histologic changes that may occur in the skin and in the internal organs and tissues. These types of effects are rarely apparent following the single-dose procedure.

The evaluation of primary irritants on animals has been found useful for relative comparisons and for predicting the concentrations that may be tolerated by man. The correlation between animals and man is not complete since we find that there is an occasional reversal. In most instances we find that the skin of the rabbit is as sensitive to primary irritants as that of man and that the relative order of irritation within a series of compounds is the same for the rabbit as for man. Here also there are occasional reversals with respect to individual compounds.

Since Landsteiner and Jacobs (8) showed that the white male guinea pig is a suitable test subject for skin sensitization studies this animal has been quite extensively used. In this laboratory we have tested several hundred substances during the past six years and feel that the results obtained on the guinea pig are extremely helpful in evaluating the relative potential sensitizing properties of a given compound when applied to the skin of man. By this technic mild sensitizing agents which will probably affect only a few individuals in a large population can readily be distinguished from those strong sensitizing agents which will affect a large proportion of the population.

The ideal test object for both primary irritants and sensitizing agents is the skin of man. In the case of the former little if any risk is run if the substances are first carefully tested on laboratory animals; however, in the case of the latter it may often be unwise and inexpedient to sensitize an individual to compounds with which he may later come in contact. For some primary irritants we have used the patch test in man in order to compare the results with those obtained on animals.

The numerical scoring system developed by Friedenwald, Hughes and Herrmann (1) for the evaluation of primary irritants using the rabbit's eye has been slightly modified and the principle extended to the development of scoring systems applicable to the evaluation of primary irritants on other mucous membranes and on the skin of animals and man. The merits of such scoring systems are presented. Finally we have tried to indicate the usefulness as well as the limitations of the techniques and procedures described in this paper for the toxicologic appraisal of a given compound or preparation intended for therapeutic, cosmetic or other topical use.

## CONCLUSIONS

Valuable qualitative and quantitative information concerning compounds and preparations intended for topical application can be obtained by the use of relatively simple procedures such as those described. In many instances some compounds and preparations can be recognized as unsafe following a few tests. The remaining ones will require much more complete investigation by these and other methods before a decision can be reached concerning the advisability of their use.

Grateful acknowledgement is made to Bert J. Vos, Jr. for many suggestions in developing the grading systems and in the development of the technique for estimation of the irritation of penile mucosa, to David W. Fassett for his contributions to the interpretation of injuries to the eye, and to Ruth B. Lang and Virginia D. Johnson for valuable technical assistance.

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# METABOLISM TOXICITY AND MANNER OF ACTION OF GOLD COMPOUNDS USED IN THE TREATMENT OF ARTHRITIS

## V A COMPARATIVE STUDY OF THE RATE OF ABSORPTION THE RETENTION AND THE RATE OF EXCRETION OF GOLD ADMINISTERED IN DIFFERENT COMPOUNDS

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Previous studies (1, 2) have indicated that following the intramuscular administration of gold compounds to the white rat gold is deposited chiefly in the liver and kidneys. Gold appeared in both the urine and feces with the more soluble compounds being excreted more rapidly. Studies in human subjects (3) however have shown that gold may be present in the urine for as long as sixteen months following its intramuscular injection, indicating a prolonged retention of the gold in the human body and its slow excretion even in the case of the more soluble compounds. Likewise the fact that gold was present in the plasma of human subjects for as long as fifteen months following its last injection indicates either a very slow absorption or a prolonged period during which the gold from the tissues slowly passes into the blood stream for excretion.

For these reasons it was considered of importance to investigate the comparative rate of absorption of gold following the intramuscular injections of various gold compounds in the white rat and the retention and excretion of gold after injections of the gold salts were discontinued.

**PROCEDURE.** Six groups of eighteen white rats (160 to 180 gms. each) were employed. Each animal was given fourteen daily intramuscular injections of 1 mg. of gold. A different gold compound (table 1) was administered to each group. Three animals of each group were decapitated on the first, eighth, fifteenth, twenty-ninth, fifty-seventh and eighty-fifth day after the last injection. The tissues listed in table 1 as well as the blood plasma were analyzed for total gold content according to the method previously described (4, 5).

**RESULTS.** The amounts of gold per mg. of tissue found at the end of various lengths of time after the last injection are listed in table 1. Each of these values is the average of the results obtained in three animals for each period. The variation of individuals for each group of animals was no greater than 5 per cent. The amount of gold in the heart and lungs is too small to be of any great significance but when present gold is retained for long periods in these tissues. Considerably more gold is present in the spleen in the case of all the compounds and its retention in this organ is likewise prolonged. The greatest percentage of

<sup>1</sup> The Rackham Arthritis Research Unit is supported by the Horace H. Rackham School of Graduate Studies of the University of Michigan.



total administered gold was found in the liver and kidneys (table 2). In all instances the gold content of these organs decreases gradually after the eighth post-injection day yet considerable quantities are still present by the eighty

TABLE 2

*Retention of gold in various tissues calculated as per cent of total injected gold*

COMPOUND		1	8	15	25	37	85
		<i>day</i>	<i>days</i>	<i>days</i>	<i>days</i>	<i>days</i>	<i>days</i>
Gold sodium thiomalate	Total body	25.20	17.00	16.85	13.05	11.10	11.05
	Carcass	13.40	7.44	9.34	6.15	4.16	5.27
	Kidney	1.81	2.90	1.15	0.78	0.77	0.36
	Liver	2.01	1.37	1.17	0.60	1.29	0.56
	Spleen	0.28	0.31	0.24	0.22	0.21	0.24
Gold sodium thiosulfate	Total body	22.70	18.50	14.51	12.45	10.21	8.95
	Carcass	11.09	8.37	5.60	5.11	5.63	5.34
	Kidney	5.41	5.17	4.13	2.84	1.28	0.87
	Liver	1.55	2.06	1.48	1.53	1.27	1.27
	Spleen	0.27	0.44	0.41	0.39	0.36	0.35
Sodium succinimido aurate	Total body	7.35	3.29	3.29	2.20	2.41	1.62
	Carcass	3.12	1.64	1.48	0.61	1.51	1.09
	Kidney	1.93	1.20	1.00	0.76	0.41	0.12
	Liver	0.74	0.29	0.47	0.36	0.33	0.29
	Spleen	0.11	0.02	0.06	0.04	0.06	0.02
Gold calcium thiomalate	Total body	46.40	35.06	23.40	16.70	17.20	13.30
	Carcass	18.35	12.08	3.49	6.05	5.03	4.01
	Kidney	3.20	2.35	1.67	0.59	1.29	0.60
	Liver	1.54	1.08	0.59	0.55	0.91	0.85
	Spleen	0.21	0.12	0.37	0.15	0.28	0.20
Gold thioglucose	Total body	42.40	23.78	21.50	20.20	21.80	15.25
	Carcass	18.86	7.46	5.66	6.42	4.39	3.32
	Kidney	4.00	3.38	1.65	0.99	1.00	0.81
	Liver	3.09	1.55	1.21	0.41	1.90	0.07
	Spleen	0.37	0.11	0.26	0.13	0.17	0.25
Colloidal gold sulfide	Total body	65.60	62.60	77.80	69.40	67.10	46.60
	Carcass	35.77	29.92	30.62	34.17	28.37	23.06
	Kidney	0.32	0.48	0.62	0.75	1.06	0.90
	Liver	7.91	3.00	2.48	3.24	3.01	2.95
	Spleen	0.22	0.04	0.04	0.03	0.04	0.04

This value was obtained by adding the amount of gold in all the viscera analyzed and the remaining carcass

fifth day. The rate at which gold is removed from the liver and spleen as well as from the heart and lung is closely parallel for each individual compound. In the case of the kidney however the actual rate at which gold is removed is much faster for all compounds except for colloidal gold sulfide. With this latter

compound there is actually more gold present on the eighty-fifth day than on the first day. We feel that the apparently more rapid rate at which gold is removed from the kidney is probably associated in part with the function of the kidney in the excretion of gold from the body as a whole. Thus, gold mobilized from other organs and passing through the kidney on the way to excretion without actually being in kidney tissue would be found as deposited gold. Likewise, the same explanation probably applies to the increased amount of gold in the kidney after the injection of colloidal gold sulfide, but modified in this instance by the very markedly slower rate of absorption and excretion. Thus, with the rapidly absorbed and rapidly excreted compounds one would anticipate the concentration of large quantities of gold in the chief excretory organ (kidney in this instance) soon after the injection of gold while at later periods after its administration there would be a correspondingly rapid decrease in the amount of gold present. Similarly, with respect to the slowly absorbed and slowly excreted compounds (for example, colloidal gold sulfide) an increasing amount of gold would tend to be found in the kidney as the period following injection increased. Gold calcium thiomalate lies intermediate between these compounds in both the rate of absorption and excretion.

The amount of gold in the leg muscle (sites of injection) is essentially a measure of the amount of unabsorbed gold. For example, the difference between the amount of gold present in the leg muscle on the first and eighth days after injection is a measure of the amount absorbed during that period. By plotting the percentages of gold absorbed by the eighth day, fifteenth day, twenty-ninth day, etc., against time, curves indicating the rate of absorption of each compound are obtained (Chart 1). The percentage absorbed in each period is calculated as a per cent of the amount present on the first post injection day. Examination of our data in this form (Chart 1) reveals that all compounds studied have a comparatively rapid initial rate of absorption after which the rate falls off to a constant, lower level. During the first eight post injection days the absorption rate is essentially the same for all compounds except gold calcium thiomalate whose initial rate is slower but continues at a higher level than does the rate of the other compounds during the ensuing twenty-one days. The comparative position of each curve on the chart also indicates the rate of absorption for each compound during the entire experimental period. Thus, sodium succinimido aurate is the most rapidly absorbed substance and colloidal gold sulfide the slowest, with the other compounds being intermediate. Comparison of Chart 1 with table 1 also reveals that the amount of unabsorbed gold on the first post-injection day is only inversely related to the rate of absorption during the eighty-five day period after injection. Thus, although the rate of absorption during the eighty-five experimental days was greatest in the case of sodium succinimido aurate, yet on the first post-injection day the least amount of unabsorbed gold was present (0.018 mg. per gm.). At the other extreme is colloidal gold sulfide, the rate of absorption of which is slowest and the amount unabsorbed on the first day the greatest (0.543 mg. per gm.).

The difference between the amount of gold present in the entire body on the

first and eighth post injection day indicates the amount excreted during that period. By calculating the amount excreted during each period (that is between the first and eighth day the first and fifteenth day etc.) and dividing this

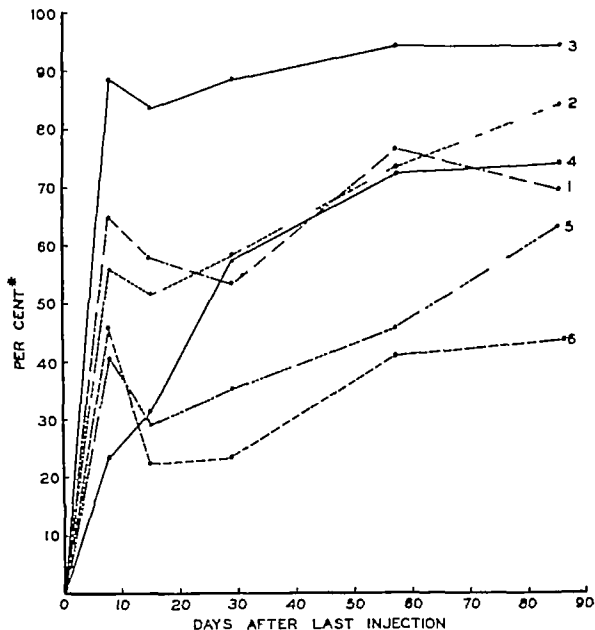


CHART 1 RATE OF ABSORPTION OF VARIOUS GOLD COMPOUNDS

Curve 1 Gold sodium thiomalate Curve 2 Gold sodium thiosulfate Curve 3 Sodium succinimido-aurate Curve 4 Gold calcium thiomalate Curve 5 Gold thio-glucose Curve 6 Colloidal gold sulfide

Percent equals the difference between the mgm of gold per gm of leg muscle on the first day and the mgm of gold per gm of leg muscle on the eighth day fifteenth day etc divided by the mgm of gold per gm of leg muscle on the first day and multiplied by 100

value by the total amount present in the body on the first day a series of values are obtained which indicate the rate of excretion of each compound (Chart 2) Examination of these data shows that there is a comparatively rapid excretory rate for all the compounds during the first eight post-injection days with a subse-

quent rapid decrease in rate. The rate of excretion for the entire period is greatest for sodium succinimido aurate and least for colloidal gold sulfide with the other compounds having intermediate values. In general, those substances

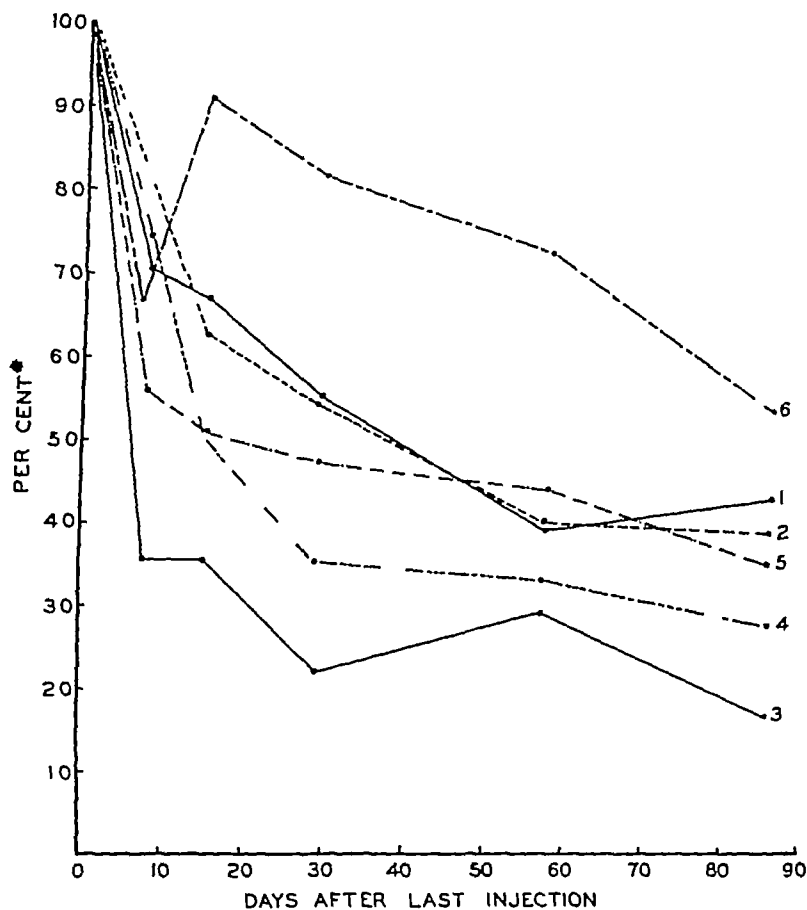


CHART 2 RATE OF EXCRETION OF VARIOUS GOLD COMPOUNDS

Curve 1 Gold sodium thiomalate, Curve 2 Gold sodium thiosulfate, Curve 3 Sodium succinimido aurate, Curve 4 Gold calcium thiomalate, Curve 5 Gold thioglucose, Curve 6 Colloidal gold sulfide

\* Per cent equals the difference between gms of gold in the entire body on the first day minus gms of gold in the entire body on the eighth day, fifteenth day, etc. divided by gms of gold in the entire body on the first day and multiplied by 100

most rapidly absorbed are most rapidly excreted

Chart 2 does not indicate the per cent of the total injected gold still not excreted by the eighty-fifth day. This is shown in table 2, and reveals that only 1.62 per cent of the injected gold is still present in the body in the case of sodium

succinimido aurate the compound with the most rapid rate of excretion whereas 46.6 per cent of injected gold is present in the body in the case of colloidal gold sulfide which has the slowest rate of excretion. This slow rate of excretion of gold sulfide is undoubtedly due in part to the extensive phagocytosis of gold particles known to occur (6).

The blood plasma gold values (table 3) at various periods after the injection of gold salts are very similar for those compounds with similar rates of absorption and excretion. The initial values are lower for the very rapidly absorbed sodium succinimido aurate (0.319 mg. per cent) and the very slowly absorbed colloidal gold sulfide (0.236 mg. per cent). All plasma values drop abruptly by the eighth day except in the case of gold calcium thiomalate which remains persistently high through the eighth day at least. This is directly correlated

TABLE 3  
*Gold content of blood plasma*

DAYS AFTER LAST INJECTION	GOLD SODIUM THIOMALATE	GOLD SODIUM THIOSULFATE	SODIUM SUCCIN- IMIDO AURATE	GOLD CALCIUM THIOMALATE	GOLD THIOGLUCOSE	COLLOIDAL GOLD SULFIDE
	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent
1	1.829	1.372	0.319	1.765	1.429	0.236
8	0.088	0.013	0.004	1.429	0.143	0.004
15	0.046	0.000	0.009	0.316	0.125	0.023
29	0.035	0.004	0.009	0.236	0.064	0.004
57	0.029	0.009	0.003	0.118	0.044	0.015
85	0.036	0.013	0.006	0.018	0.029	0.011

with the difference in the absorption rate of this substance (Chart 1). In general, the blood plasma values are correlated with rate of absorption during any period. This correlation is not strictly exact but applies in general. With all compounds a small amount of gold is still present in the plasma eighty-five days after the last injection.

#### SUMMARY

1. Gold is retained in the liver, spleen, and kidneys in significant quantities for as long as eighty-five days after its intramuscular injection in rats. The rate of removal of gold from these tissues is essentially parallel for each compound except from the kidney where it is influenced by the excretory function of that organ.

2. The absorption of gold compounds after intramuscular injection is incomplete eighty-five days after the last injection. The rate of absorption is most rapid in the case of sodium succinimido aurate and slowest in colloidal gold sulfide with gold sodium thiomalate, gold sodium thiosulfate, gold calcium thiomalate, and gold thioglucose having intermediate rates.

3. The rate of excretion of gold is parallel to the absorption rate for each compound but is incomplete eighty-five days after the last injection even in the case of sodium succinimido aurate which is the most rapidly excreted substance.

4 Gold is present in blood plasma throughout the eighty-five days after the last injection when our observations ceased

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